



Monocytes and their role in inflammation following Cardiopulmonary Bypass.

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ABSTRACT

Over the last decade there have been dramatic improvements in paediatric cardiac surgery and the outcome for children with congenital heart disease. Whilst the majority recover promptly from surgery, a small unpredictable group of children have persistent requirements for ventilatory and intensive care support. For these children sepsis and systemic inflammatory response syndrome (SIRS) appears to be a major cause of morbidity and mortality. Antigens of the major histocompatibility complex type II (MHC Class II) are decreased on circulating monocytes of many critically ill patients. Persistently low expression of these antigens has been associated with poor prognosis and increased susceptibility to infection. The work presented here examines the hypothesis that '*monocyte deactivation*', as indicated by reduced MHC Class II expression and decreased whole blood secretion of pro-inflammatory cytokines in response to lipopolysaccharide (LPS), is a factor in the development of sepsis/SIRS following cardiopulmonary bypass.

Using flow cytometry, confocal microscopy, enzyme-linked immunosorbent assays, *ex vivo* whole blood stimulation, and real time-polymerase chain reaction it has been shown that (1) monocyte surface expression of MHC Class II falls following cardiac surgery involving CPB; (2) this reduction in surface expression is paralleled by a reduction in intracellular MHC Class II stores, and increased transcription of MHC Class II related genes; (3) bypass results in the early and simultaneous rise in both pro- and anti-inflammatory cytokines; (4) whole blood drawn from patients following CPB is profoundly hyporesponsive to LPS stimulation; (5) whole blood hypo-responsiveness precedes the rise in circulating cytokine and that (6) the inflammatory response and morbidity following CPB may be influenced by the presence of cytokine polymorphisms.

The results shown here strongly suggest that monocyte MHC Class II surface expression is an excellent diagnostic tool for the identification of the subgroup of children most likely to develop post-operative complications. By identifying this subgroup of children immunomodulative therapy can be appropriately targeted to restore homeostasis and improve outcome.

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It is a great pleasure to have a profession where not a day passes that I don't learn something new. Undertaking a PhD has opened my eyes to the fact that I didn't really understand many of the clinical occurrences I observed and attempted to manipulate. It has also made me aware as a doctor, that many of the things we credit ourselves with achieving when managing patients are probably being achieved by the homeostatic mechanisms that exist within the body. Despite this glimpse into the extraordinary amount of new information that is being gleaned everyday in science, the art of medicine is fundamental to its successful practise and a truly great doctor. I would like to thank Professor Nigel Klein, my principal supervisor, who practises this art with such energy and enthusiasm it is hard not to be swept along with the tide.

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Meredith
December 2003.

DECLARATION

The work presented in chapters 4 and 5 was carried out as part of a collaborative study with Dr Andreas Hoschitzky from the department of Cardiothoracic Surgery at Great Ormond Street Hospital, London. In chapter 4 Dr Hoschitzky measured all cardiac indices during the first 24 hours post-operatively. Blood samples and outcome data used in chapters 4 and 5 were collected jointly. All laboratory work, statistical analysis, and discussion are solely the work of the author.

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Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
Bp	Base pair
BSA	Bovine serum albumin
CI	Confidence interval
CHD	Congenital heart disease
CICU	Cardiac intensive care unit
CIITA	MHC Class II transactivator
CPB	Cardiopulmonary bypass
DNA	Deoxyribonucleic acid
EDTA	Ethenediamine tetraacetic acid
ELISA	Enzyme linked immune sorbant assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluoroscein isothiocyanate
GM-CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen
ICU	Intensive care unit
IFN- γ	Interferon gamma
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IMS	Industrial methylated spirits
iNOS	Inducible nitric oxide synthase
kDa	Kilo Daltons
LOS	Length of Stay
LPS	Lipopolysaccharide endotoxin
M	molar
mAb	monoclonal antibody
mfi	Median fluorescence intensity
MHC	Major histocompatibility antigen
MIIC	MHC Class II compartment

mRNA	Messenger RNA
MUF	Modified ultrafiltration
NF- κ B	Nuclear factor kappa B
NO	Nitric oxide
OD	Optical density
OR	Odds ratio
p	Probability
PCR	Polymerase chain reaction
PBMC's	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PIM	Paediatric index of mortality
Pre op	Preoperative
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Real time-polymerase chain reaction
SEM	Standard error of the mean
SIRS	Systemic Inflammatory response syndrome
SNP	Single base-pair substitution
sTNFR	Soluble tumour necrosis factor receptor
$t_{1/2}$	half life
TLR	Toll-like receptors
TGF- β	Transforming growth factor beta
TNF- α	Tumour necrosis factor alpha
X-clamp	Aortic cross clamp

1.1 CONGENITAL HEART DISEASE

Congenital heart disease (CHD) is the most common of all major birth defects, occurring in 0.8 to 1% of live born children (Table 1.1). The incidence is higher in stillborn infants and spontaneous abortions. For paediatricians and parents, the last 30 years has seen a dramatic change in the diagnosis, management and outcome for an infant with a congenital heart defect. The prognosis has changed from almost certain death, to the opportunity for complete surgical repair with a low risk of mortality.

Cardiac Lesions	(Incidence per 10 000 live births)
Ventricular septal defect	30-50
Patent ductus arteriosus ^a	10
Atrial septal defect (secundum)	7
Endocardial cushion defect ^b	3-5
Coarctation of the aorta	6
Aortic stenosis	5
Pulmonary stenosis	7
Tetralogy of Fallot	5
Transposition of great arteries	5
Pulmonary atresia	1-2
Tricuspid atresia	1-2
Truncus arteriosus	1
Total anomalous pulmonary venous connection	1
Aortic atresia	1
Double outlet right ventricle	<1
Atrial isomerism	2
Single ventricle	1
Ebstein's malformation	<1

Table 1.1 Incidence of common types of congenital heart defects at birth.
Ranges are a reflection of variation in different case reports.

^aIncidence excludes pre-term infants. ^bIncidence excludes Trisomy 21
(Adapted from Rudolph's *Pediatrics* 19th edition, 1991)

1.1.1 Development of Paediatric Cardiac Surgery

In order for complex surgery to take place on the heart or great vessels, a mechanical device is required that can temporarily take over the function of the heart and lungs. This provides the surgeon with a bloodless, motionless field. From the 1930's to the 1960's progress in paediatric cardiac surgery paralleled progress in the development of cardiopulmonary bypass (CPB).

In 1937 John Gibbon built the first heart-lung machine using an animal subject. A roller pump was used as a substitute for the heart, and anticoagulated blood was directly exposed to oxygen as it dripped along wires on a vertically mounted screen. It was too small for humans and most animals died from haemolysis, air embolism or "inflammation". The following year Robert Gross successfully ligated the patent arterial duct (PDA), ushering in the era of surgery for congenital heart disease (Gross and Hubbard, 1939). In 1946 Gibbon joined with engineers to invent a heart-lung machine, which minimised haemolysis and involved a closed circuit, preventing entry of air bubbles. About the same time Alfred Blalock and Helen Taussig developed a subclavian to pulmonary artery shunt, whilst doctors in Sweden carried out the first successful repair of an aortic coarctation (Blalock and Taussig, 1945; Crafoord and Nylin, 1945).

By the late 1940's Bill Bigelow was performing open-heart surgery on dogs using cooling blankets and ice bags. By cooling the dog to 20°C he was able to decrease oxygen consumption significantly allowing isolation of the heart from circulation for 15 minutes operating time. Within 6 years John Lewis utilised immersion hypothermia to close a paediatric atrial septal defect (ASD). In 1953 the first heart-lung machine was used in human cardiac surgery (Lillehei, Cohen, and Warden, 1955). By 1958 adult cardiac surgeons were utilising cardiopulmonary bypass, deep hypothermia and circulatory arrest to assist them in their repairs of cardiac defects. However it was not until the 1970's, that these techniques were utilised in children (Barratt-Boyes, 1973).

Over the last two decades, cardiopulmonary bypass has been successfully used in the correction of congenital heart disease in children. Initially only older children

underwent a primary repair, with young children and infants having simpler palliative procedures. Gradually with increasing experience, a transition has been made from palliative to corrective surgery even in very small infants. Developments in cardiopulmonary bypass have been crucial to this transition in patient management.

1.2 CARDIOPULMONARY BYPASS

The basic procedure involved in CPB is straightforward. Prior to placing the child or infant on bypass, a median incision through the sternum to expose the anterior mediastinum and pericardium is required. The bypass machine (Fig 1.1) is primed with a balanced electrolyte or blood solution. Venous blood is drained by gravity through a cannula placed in the right atrial appendage or by two cannulae placed in the inferior and superior vena cavae (Fig 1.2). Oxygenated blood is returned to the patient via another cannula placed in the ascending aorta.

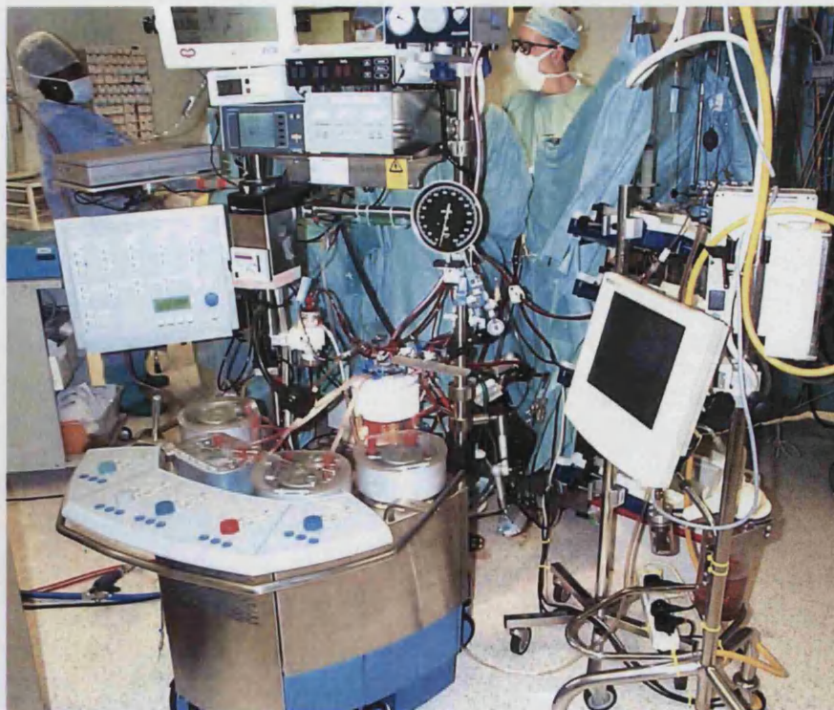


Figure 1.1 Cardiopulmonary Bypass machine. Cardiopulmonary bypass in theatre is pictured. Blood from the patient can be seen entering and exiting the bypass machine through plastic tubing.

Gas exchange takes place across a membrane oxygenator, and blood temperature can be adjusted by the use of a heat exchanger. The patient is anticoagulated for the duration of bypass. Depending on the surgery undertaken, the heart may be allowed to beat during bypass, or be temporarily arrested by the administration of cardioplegia solution.

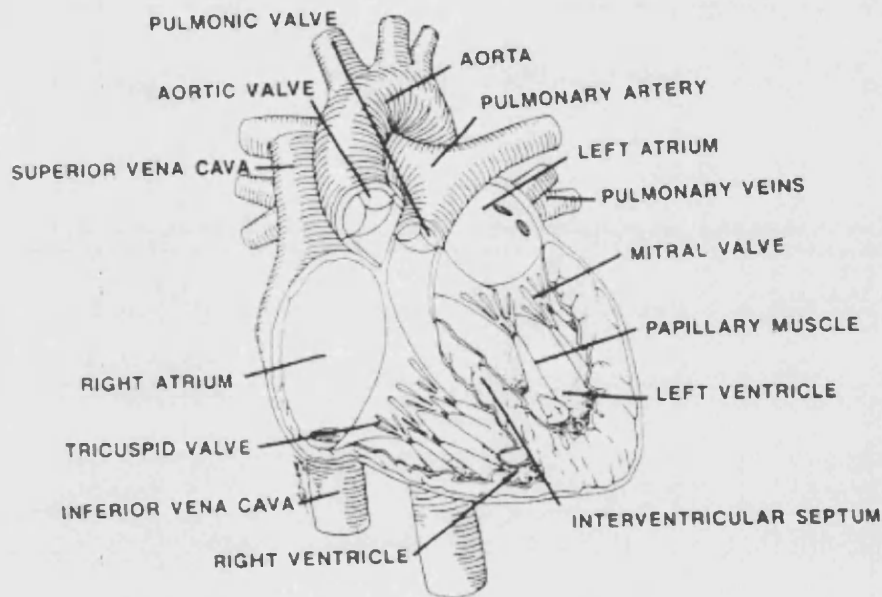


Figure 1.2 Basic anatomy of the heart. (*Grants anatomy 10th edition*)

1.2.1 CPB and Outcome

Over the past 30 years there has been a progressive reduction in the mortality associated with repair of congenital heart defects (Fig 1.3). The reasons for this are complex, but a combination of increased surgical expertise, technological advancements and improved post-operative care have all played an important role. As mortality has decreased however, there has been an unmasking of the degree of morbidity associated with CPB. Data from the Society of Thoracic Surgeons National Database report 20% of 'low-risk' adult patients develop postoperative complications following cardiac surgery (Grover,

1999). The incidence of multiple organ dysfunction syndrome (MODS) following CPB was 11% with a mortality rate of 41% (Kollef, Wragge, and Pasque, 1995).

When death was the natural outcome associated with a congenital cardiac defect, parents and doctors were accepting of any morbidity associated with a successful surgical repair. However as survival has increased there has been a reduction in both the professional and lay communities acceptance of complications. There has therefore been an increasing interest in the reasons why CPB is associated with significant morbidity.

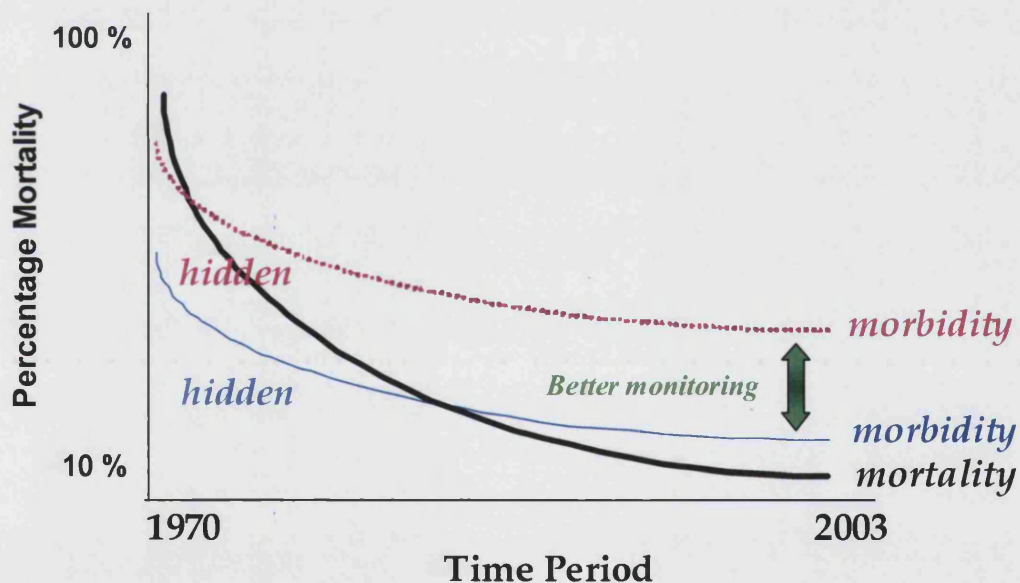


Figure 1.3 Schematic diagram of outcome for patients undergoing surgery for Congenital Heart Disease. With improvement in cardiac surgical techniques and post-operative care there has been a rapid reduction in mortality associated with surgery but an unmasking of morbidity. Advances in monitoring of end-organ functioning have suggested that morbidity is probably being underestimated.

1.2.2 The inflammatory response to CPB

A consistent observation in patients undergoing CPB was that a high proportion of patients had features of a systemic inflammatory response. This was particularly noticeable in the early post-operative period and included clinical features such as capillary leak, oedema, and organ dysfunction. This was termed '*post-perfusion syndrome*'. The aetiology of this syndrome was not known but felt to be due to a combination of the processes of CPB and cardiac surgery. The surgical insult, exposure of blood to foreign materials as well as fluctuations in temperature, fluids, coagulation and tissue perfusion were recognised as potential causes of this syndrome (Fig 1.4). It was felt likely that these events mediated the *post-perfusion syndrome* through activation of humoral and cellular pathways.

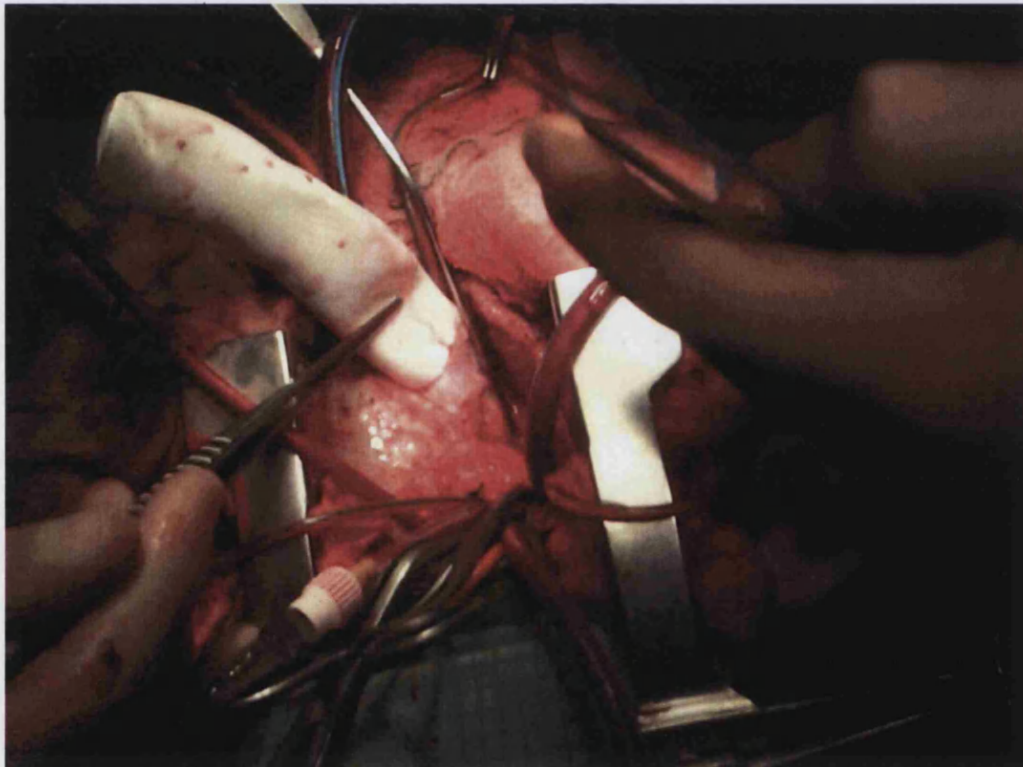


Figure 1.4 The surgical field during paediatric cardiac surgery.

During cardiac surgery involving CPB the patient is exposed to numerous pro-inflammatory triggers including local tissue trauma, foreign bodies, and exposure of blood to foreign surfaces.

More recent data has suggested that *post-perfusion syndrome* can be mimicked in animal models by the infusion of pro-inflammatory cytokines and components of the complement pathway (Pagani, Baker and others, 1992; Tracey, Beutler and others, 1986; Yokoyama, Vaca and others, 1993). This led to attempts to improve the outcome of patients on CPB by blocking individual mediators or even whole inflammatory cascades. To date these have failed to live up to expectations (Bouter, Schippers and others, 2002; Chaney, 2002; Harig, Feyrer and others, 1999; Wan, LeClerc, and Vincent, 1997b). There are still many gaps in our understanding of the inflammatory response to CPB and in particular the detailed timing of individual cytokine production and release and how they interact with each other remains sketchy. Perhaps ignored in early studies, has been the recognition that mediators which act to oppose pro-inflammation may themselves be playing a role in the pathology associated with CPB.

1.2.2.1 Cardiopulmonary Bypass and Pro-inflammation

The division between pro and anti-inflammatory mediators is simplistic as many mediators have properties that correspond to both categories. However for the purposes of this introduction, cytokines will be discussed under these two headings.

Pro-inflammatory triggers during CPB include:

- * exposure of blood to foreign surfaces (bypass circuit)
- * ischemia-reperfusion injury to brain, heart, lungs, kidneys, and liver following aortic cross clamping
- * endotoxaemia during the procedure (Berendes, Mollhoff and others, 1997; Mollhoff, Loick and others, 1999; Jansen and others, 1995)
- * hypothermia
- * tissue trauma
- * blood loss or transfusion.

(Roach, Kanchuger and others, 1996) (Martinez-Pellus, Merino and others, 1997)

These trigger release of mediators including tumour necrosis factor (TNF- α), interleukins (IL)-1, -6, and -8, products of the complement cascade, free oxygen radicals, acute phase reactants, and increased cellular expression of the adhesion molecules L-selectin, CD11b and CD18 (Butler, Rocker, and Westaby, 1993) (Kirklin, Westaby and others, 1983) (Fig 1.5).

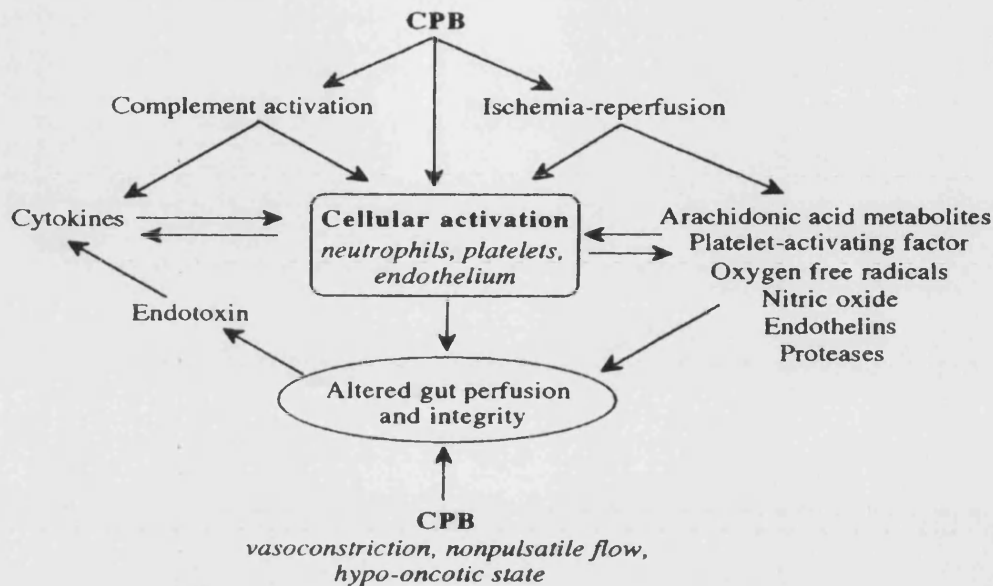


Figure 1.5 A schematic representation of the inflammatory response to CPB (adapted from Wan S et al 1998). CPB causes both direct and indirect activation of the immune system, many components of which feed back to further amplify the inflammatory response.

1.2.2.2 Cardiopulmonary Bypass and Anti-inflammatory Mediators

Along with the release of pro-inflammatory cytokines, CPB stimulates a phased anti-inflammatory response, involving anti-inflammatory cytokines, soluble cytokine receptors, and cytokine receptor antagonists (McBride, Armstrong and others, 1995; McBride, Armstrong and others, 1996). Key anti-inflammatory cytokines include interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1ra), TNF soluble receptors 1 and 2 (sTNFR-1 and 2), and transforming growth factor β (TGF- β). These anti-inflammatory cytokines are important in controlling the pro-inflammatory response initiated by the surgical insult, and it has been suggested that the clinical prognosis following CPB may depend on the balance between pro and anti-inflammatory cytokines (McBride, Armstrong and others, 1995).

Numerous studies of the inflammatory response to CPB have implicated the cytokine network in the pathogenesis of *post-perfusion syndrome*, however a direct cause-and-effect relationship has not been demonstrated (Casey, 1993; Wan and Yim, 1999). In particular, inflammatory cytokines have been shown to play a critical role in inducing myocardial injury. Before discussing the role of cytokines in morbidity following cardiac surgery and cardiopulmonary bypass, it is appropriate to outline the physical and chemical characteristics of the key cytokines.

1.2.3 Physical and chemical characteristics of cytokines implicated in post-operative morbidity

1.2.3.1 Tumour necrosis factor (TNF- α)

1.2.3.1.1 *Structure*

TNF- α is a 26-kDa transmembrane protein which is cleaved by a site specific metalloprotease (TNF- α converting enzyme) to generate the soluble 17-kDa form (Gearing, Beckett and others, 1994). Most biological activity of TNF- α is attributed to its soluble form, however membrane bound TNF does have some activity, and may be more able to bind and activate the TNF receptor 2 than its soluble form (Grell, Douni and others, 1995).

1.2.3.1.2 *TNF- α production*

Activated monocytes, macrophages, T and B-cells, NK cells, mast cells and fibroblasts secrete TNF- α . The biosynthesis of TNF- α is tightly controlled. Secretion is predominantly from activated cells, with low or undetectable levels of TNF- α from resting cells (Beutler, 2000; Beutler, Krochin and others, 1986). It is one of the major pro-inflammatory cytokines produced by monocytes after stimulation with lipopolysaccharide (LPS). Despite this, TNF- α has a short biological half life and circulating levels of TNF- α are short lived in the plasma even in the continued presence of endotoxin (Fong and Lowry, 1990). The presence of some cytokines such as interferon gamma (IFN- γ), granulocyte-macrophage colony-stimulating factor (GM-CSF) can increase production of TNF- α by monocytes, whilst other cytokines, such as

IL-10, IL-4, IL-13, suppress its production (de Waal, Abrams and others, 1991; Dickensheets, Freeman and others, 1997; Hart, Whitty and others, 1989). TNF- α secretion can be influenced further by genetic variability in the promoter region of the TNF- α gene in humans. This is discussed in section 1.3.1.2.

1.2.3.1.3 *TNF receptors*

There are two TNF- α receptors, TNFR-1 and TNFR-2. These are members of the *TNF receptor superfamily* which is defined by the presence of four cysteine-rich repeats in the extracellular region. The TNF receptor superfamily includes both cytokine receptors and other leukocyte surface glycoproteins such as CD40 and FAS antigen. Some of the receptors within this family bind more than one ligand. TNFR-1 and TNFR-2 bind both TNF- α and TNF- β with high affinity despite their large differences in amino acid sequence.

There is less than 25% homology between TNFR-1 and TNFR-2, with structurally different intracellular domains indicating different signalling mechanisms. The TNF receptors are present on all cells except for red blood cells. It is generally believed that TNFR-1 is responsible for the majority of biological actions of TNF- α . However cells of the immune system tend to express TNFR-2.

Under most circumstances TNFR-1 signals through protein kinases, such as I kappa B (I- κ B) kinase, to increase pro-inflammatory gene expression. The central pro-inflammatory transcription factor appears to be nuclear factor kappa B (NF- κ B). TNFR-1 can also signal through a series of protein-protein interactions to activate caspase-8 or caspase-2, resulting in apoptotic cell death. Far less is known about the signalling mechanism of TNFR-2 (Fig 1.6).

Soluble forms of both TNF receptors can be derived from the extracellular domains of the membrane bound receptor. Activation of monocytes by endotoxin or TNF- α induces rapid shedding of membrane bound TNFR-2 molecules, and internalisation of TNFR-1 (Ding, Sanchez and others, 1989; Higuchi and Aggarwal, 1994). Binding of only 1-5% of cell surface TNF- α receptors with TNF- α can induce shedding of soluble receptors from the entire cell surface (Curtis, McAtear and others, 1995). In contrast to TNF- α , sTNFR-2 molecules are stable with relatively long half-lives. Both agonist and

antagonist actions have been attributed to the soluble forms of the receptor. When sTNFR levels are high, they act to inhibit TNF- α by competing for ligand with the membrane bound TNFR (Van Zee, Kohno and others, 1992). However when soluble receptor concentrations are low, their presence may enhance activity by stabilizing the TNF- α molecule and prolonging its availability to bind to surface receptors (Aderka, Engelmann and others, 1992; Mohler, Torrance and others, 1993).

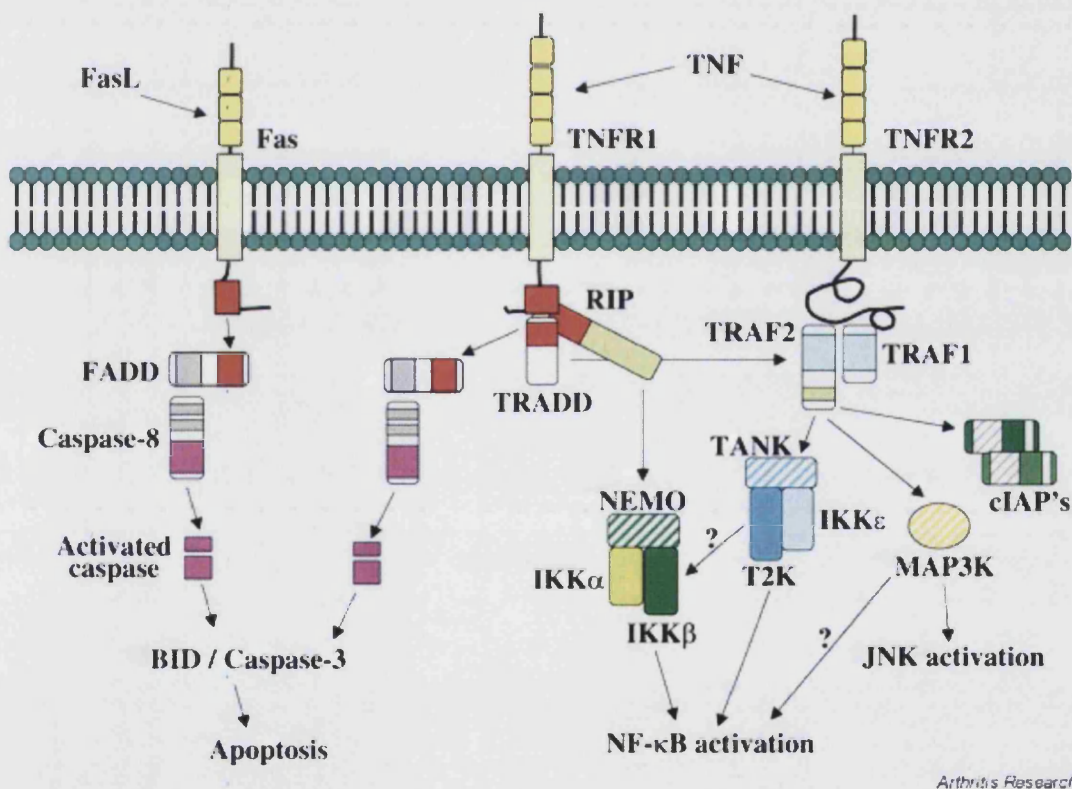


Figure 1.6 The intracellular signalling pathways downstream of TNF receptors. The TNFR-1, TNFR-2 and Fas, receptors are shown with their transmembrane domains. Various adaptor proteins and signal transducing molecules that convey the signal initiated by TNF- α binding to its receptor are shown, as are the cross talk between molecules and pathways. (from *Arthritis-Research*)

1.2.3.1.4 *Biological Effects of TNF- α*

1.2.3.1.4.1 *Systemic*

Circulating levels of TNF- α are associated with a rise in body temperature, anorexia, and release of acute phase proteins from the liver. TNF- α has a number of effects on the microvascular endothelium, which on the local level are important in host defence, but on a systemic level can be very harmful. TNF- α increases blood flow and vascular permeability. It increases endothelial adhesiveness to WBC and platelets via increased expression of tissue factor and adhesion molecules. Locally these act to limit the spread of bacteria, however if there is systemic overflow, then these same effects on blood vessels will result in disseminated intravascular coagulation (DIC) and shock.

1.2.3.1.4.2 *Monocytes / Macrophages*

TNF- α acts synergistically with IFN- γ in the activation of monocytes/macrophages, resulting in induction and secretion of many cytokines including IL-1, IL-6, IL-8, GM-CSF, IFN- γ , TGF- β , IL-10, and prostaglandin E₂. It also acts to induce further TNF- α secretion. It encourages migration of dendritic cells from the periphery to local lymph nodes where maturation takes place. This results in increased antigen presentation that is central to the link between innate and adaptive immunity.

1.2.3.1.4.3 *T & B cells*

It promotes differentiation and activation of T & B-cells, and induction of apoptosis in mature T-cells.

1.2.3.2 Interleukin 6 (IL-6)

1.2.3.2.1 *Structure*

IL-6 is a 30-kDa glycoprotein with a four anti-parallel α -helical structure containing intramolecular disulphide bonds similar to IL-2, IL-4, GM-CSF (Somers, Stahl, and Sehra, 1997).

1.2.3.2.2 *IL-6 production*

IL-6 is produced at local sites and released into the circulation in almost all situations where normal homeostasis is disturbed. IL-6 is usually induced in combination with TNF- α and IL-1, and together these cytokines are responsible for the induction of fever, corticosterone release, and the hepatic proteins that constitute an acute phase reaction. IL-6 is secreted by T-cells, B-cells, monocyte/macrophages, bone marrow stromal cells, fibroblasts, keratinocytes, adipocytes, astrocytes, myocytes and endothelial cells. IL-6 production is used in some studies as a correlate of cellular activation.

Compared to the major acute increases observed in inflammation, modest chronic elevations in circulating levels of IL-6 have also been observed in obesity, cardiovascular disease, and Type-2 diabetes (Hotamisligil, Arner and others, 1995; Yudkin, Kumari and others, 2000). Low levels of circulating IL-6 can also be found in the blood of normal individuals.

1.2.3.2.3 *IL-6 receptor*

The IL-6 receptor belongs to the *Class I cytokine receptor superfamily* and is formed by the noncovalent association of two subunits (gp80 and gp130). The IL-6R α -chain (gp80) binds the IL-6 molecule with low affinity but does not signal. The β -chain (gp130) does not bind IL-6 directly, but associates with the IL-6/ α -chain complex and is responsible for signal transduction through the gp130 subunit (Taga, Hibi and others, 1989). The IL-6 receptor is expressed on activated B cells, plasma cells, T cells, monocytes, epithelial cells, fibroblasts, hepatocytes and neural cells (Fitzgerald, O'Neill and others, 2001).

Soluble forms of the IL-6 receptor are found in the blood of normal adult controls. Circumstances surrounding the generation of the soluble molecule are not clear. The soluble gp130 subunit lacks the transmembrane segment, and has been shown to have an inhibitory action on circulating IL-6. In contrast, the soluble form of the gp80 subunit has been shown to bind circulating IL-6 and extends its half-life (1998).

1.2.3.2.4 *Biological Effects of IL-6*

1.2.3.2.4.1 *Systemic*

Of all cytokines, IL-6 has been found most consistently in the circulation of septic patients (Hack, De Groot and others, 1989). It is unclear however whether IL-6 is merely an acute phase reaction-inducing cytokine or has any additional pro-inflammatory or anti-inflammatory actions. Serum IL-6 levels have been shown to correlate with C-reactive protein levels, fever, and mortality of patients with sepsis or trauma, but it is difficult to separate the biological effect of IL-6 from the degree of tissue injury that is stimulating the production of IL-6 (Hack, De Groot and others, 1989; Holzheimer, Capel and others, 2000; Patel, Deen and others, 1994; Schluter, Konig and others, 1991).

1.2.3.2.4.2 *Monocytes / Macrophages*

IL-6 is secreted by monocytes following endotoxaemia, trauma, and acute infections. It has a negative feedback effect which inhibits further pro-inflammatory cytokine secretion (see below). IL-6 can be used to induce macrophage differentiation.

1.2.3.2.4.3 *T & B cells*

IL-6 is important in T-cell proliferation and differentiation. The effects of IL-6 on T-cells are synergistic with IL-1 and TNF- α . In addition IL-6 produced by T-cells and macrophages following antigen presentation, stimulates B cells to proliferate and differentiate into antibody producing cells.

1.2.3.2.5 *Anti-inflammatory properties of IL-6*

Recently the anti-inflammatory properties of IL-6 have been recognised. In IL-6 knockout mice both local and systemic acute inflammatory responses are more marked, (Xing, Gauldie and others, 1998). IL-6 has been shown to inhibit the synthesis of TNF- α and IL-1, promote the synthesis of IL1-ra and sTNFR, whilst having little effect on the synthesis of the anti-inflammatory cytokines IL-10 or TGF- β (Libert, Takahashi and others, 1994; Xing, Gauldie and others, 1998).

1.2.3.3 *Interleukin-1 (IL-1)*

1.2.3.3.1 *Structure*

There are 3 members of the IL-1 family; IL-1 α , IL-1 β , and interleukin 1 receptor antagonist protein (IRAP or IL-1ra). IL-1 α and IL-1 β are synthesised as large precursor molecules, but in contrast to other proteins, the synthesis occurs in association with microtubules, not in the endoplasmic reticulum (Fig 1.7) (Stevenson, Torrano and others, 1992). These precursor molecules have been shown to lack the series of amino acids (leader sequence) that enable the precursor protein to be inserted into the Golgi for cleavage, packaging and transport out of the cell. Instead the large precursor molecules remain intracellular or are expressed on the cell surface membrane where they are cleaved by proteases. Both the cytosolic and membrane bound IL-1 α precursor are biologically active (Brody and Durum, 1989). Unlike IL-1 α , the IL-1 β precursor requires cleavage for biological activity. The IL-1 β precursor is cut intracellularly by a specific IL-1 β converting enzyme (ICE) (Cerretti, Kozlosky and others, 1992). The mature biologically active molecule is then secreted through membrane channels.

1.2.3.3.2 *IL-1 Production*

IL-1 α and β are made mainly by monocytes and macrophages, but also endothelial cells, fibroblasts and epidermal cells. Production is stimulated by LPS, other microbial products, cytokines (TNF- α , IFN- γ , IL-2, GM-CSF), T-cell/antigen presenting cell interactions, and immune complexes (Stylianou and Saklatvala, 1998).

1.2.3.3.3 *IL-1 Receptor*

There are two IL-1 receptors. The type I receptor (IL-1RI), is ubiquitously expressed, binds both IL-1 α and IL-1 β with equal avidity, and transduces the IL-1 signal. The cytoplasmic tail of the type I receptor contains domains that are homologous with the *Toll* receptors. Binding of IL-1 signals the activation of a series of kinase cascades very similar to those activated by TNF- α (Stylianou and Saklatvala, 1998).

The type II receptor (IL-1RII) is more limited in its expression, being found on monocytes, B-cells, and neutrophils, and preferentially binds IL-1 β . IL-1RII does not transduce a signal, but is shed from the surface on cellular activation, and in the soluble form binds IL-1 β with high affinity, inhibiting the cytokines action.

1.2.3.4.3 *Biological effects*

Increased IL-1 production has been reported in various disease states including infection, solid tumours, leukaemias, autoimmune disorders, graft versus host disease, following trauma and myocardial infarction, and in healthy subjects after strenuous exercise (Dinarello, 1996). However, most processes that cause production of IL-1 will also cause production of IL-6 or TNF- α , making it difficult to ascribe any particular finding specifically to IL-1. IL-1 stimulates hepatic acute-phase protein synthesis and is a major endogenous pyrogen. IL-1 acts on the hypothalamic-pituitary axis to induce corticosteroid release. On vascular endothelium, IL-1 increases expression of adhesion receptors causing leukocyte accumulation and chemokine secretion.

Whilst cells express a large number of IL-1RI, they require only a small number of receptors to bind IL-1 for cell stimulation (3-5 receptors per cell) (Gabay, 2002).

1.2.3.4 Interleukin-1 receptor antagonist (IL-1ra)

1.2.3.4.1 *Structure*

This is the 3rd member of the IL-1 family; and recognised predominantly as an anti-inflammatory cytokine. In direct contrast to IL-1, IL-1ra is a glycosylated protein (22 kDa) that is synthesized in the endoplasmic reticulum, processed in the golgi, and actively secreted from the cell (Fig 1.7C)(Andersson, Bjork and others, 1992).

1.2.3.4.2 *IL-1ra Production*

IL-1ra is secreted predominantly by monocytes and macrophages, but also by neutrophils, hepatocytes, microglial cells, epithelial cells fibroblasts, and keratinocytes (Arend and Guthridge, 2000; Stylianou and Saklatvala, 1998). Production of IL-1ra follows the production of IL-1 by 1 to 2 hours, and may be stimulated by it. Compared to expression of IL-1 β , IL-1ra mRNA levels remain elevated for several hours following stimulation. Soluble IL-1ra binds to both IL-1 receptors with an avidity equal to IL-1 α and β , but does not induce receptor internalisation or intracellular signalling (Dinarello, 1996).

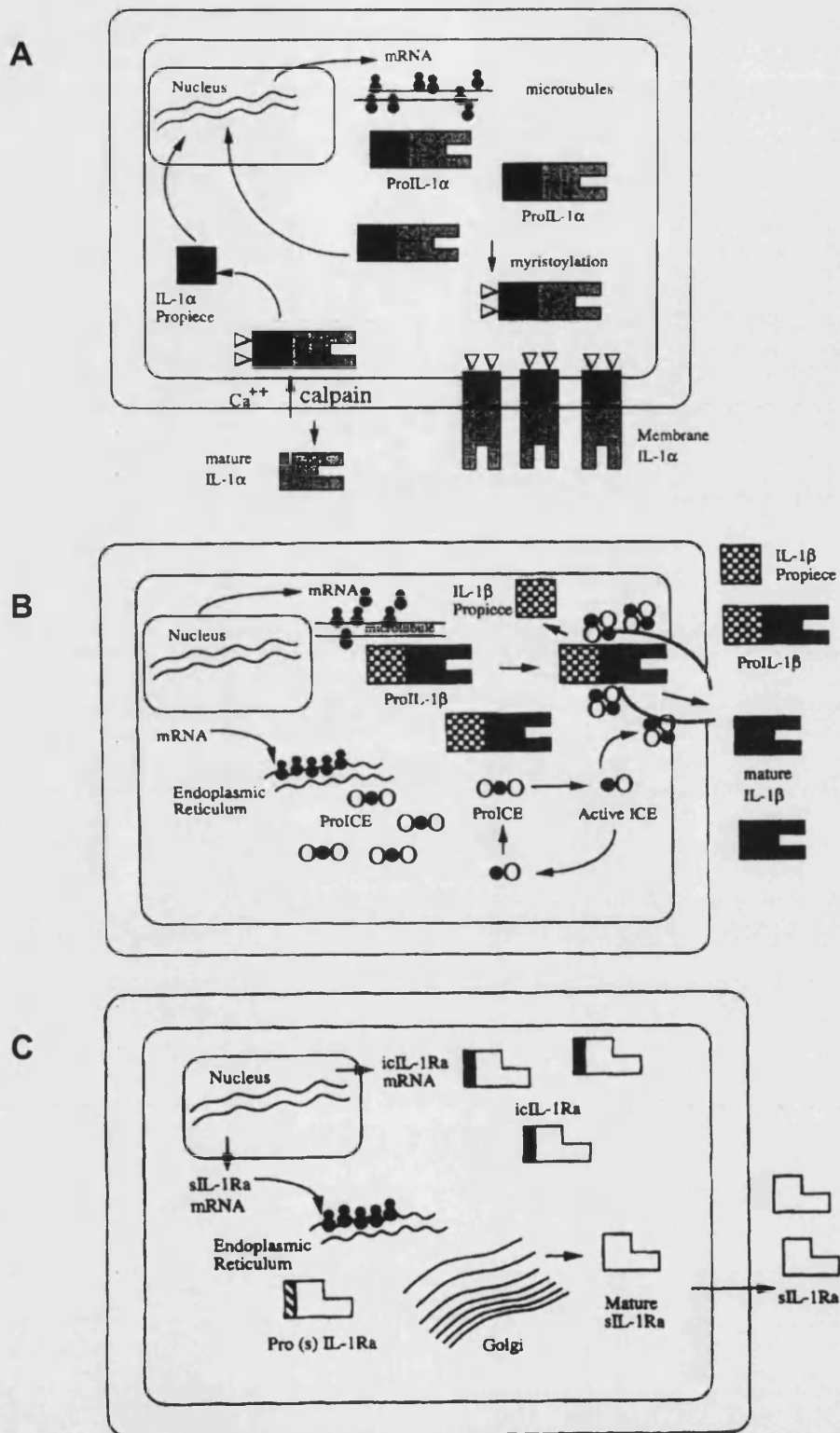


Figure 1.7 Monocyte production of IL-1α (A), IL-1β (B), and IL-1ra (C). mRNA coding for both IL-1α and IL-1β is translated in association with microtubules. (A) The majority of pro-IL-1α remains in the cytosol but about 10-15% is transported to the cell membrane. Both the intracellular and membrane bound forms are biologically active. The pro-IL-1α can be cleaved and released extracellularly by the cysteine protease, calpain. (B) After synthesis the pro-IL-1β remains primarily cytosolic until cleaved by ICE and transported out of the cell by membrane channels. A small amount of pro-IL-1β is secreted prior to cleavage. Pro-IL-1β has limited biological activity. (C) When stimulated, monocyte pro-IL-1ra is translated in the endoplasmic reticulum and transported to the golgi. The leader sequence is cleaved and the soluble form of IL-1ra is secreted from the cell. (Adapted from Dinarello CA et al 1996)

1.2.3.4.3 *Biological effects*

IL-1ra competitively inhibits the binding of IL-1 to the cell surface receptors. However because of the extreme sensitivity of cells to the presence of even small concentrations of IL-1, IL-1ra levels need to be in vast excess to effectively inhibit cellular activation.

Endogenously produced IL-1ra is important in limiting inflammation and tissue damage in animal models of diseases such as arthritis, immune complex lung injury, graft versus host disease, septic shock, and ischemia/reperfusion (Arend and Guthridge, 2000). In studies of human disease, there has been some suggestion that the level of production of IL-1ra in local tissues may not be sufficient to effectively block the inflammatory affect of IL-1 production (Arend and Guthridge, 2000). Despite its actions, recombinant IL-1ra has failed to show any benefit in human inflammatory trials to date (Arend, Malyak and others, 1998).

In addition to the soluble form of IL-1ra, there are 3 intracellular isoforms, the biological functions of which are less clear.

1.2.3.5 Interleukin-10 (IL-10)

1.2.3.5.1 *Structure*

Interleukin-10 is an 18-kDa polypeptide, and the only cytokine considered to have a purely anti-inflammatory action. It is a member of the four α -helix family of cytokines. Human IL-10 is stable in basic conditions (up to pH 11) but rapidly inactivated by exposure to acid (pH <5.5).

1.2.3.5.2 *IL-10 production*

IL-10 is readily measurable in the circulation of patients with systemic illnesses and a variety of inflammatory states (Marchant, Deviere and others, 1994; van der, de Waal and others, 1997). It is secreted by monocytes/macrophages, T cells, activated B cells, and keratinocytes (Moore, de Waal and others, 2001; Moore, O'Garra and others, 1993), and considered one of the most important anti-inflammatory cytokine in humans (Opal and DePalo, 2000). It plays a major role in the regulation of immune response by inhibiting monocyte/macrophage, NK cells, and T cell effector function.

1.2.3.5.3 *IL-10 receptor*

The IL-10 receptor belongs to the *interferon receptor superfamily*, and is a single spanning transmembrane glycoprotein. Signal transduction by this receptor involves phosphorylation and activation of tyrosine kinases (Jak1) and transcription factors STAT1a & 3 (Fitzgerald, O'Neill and others, 2001). IL-10 receptors are constitutively expressed on most haemopoietic cells, and can be induced on non-haemopoietic cells such as fibroblasts and keratinocytes (Moore, de Waal and others, 2001). There is no literature describing a soluble form of the IL-10 receptor.

1.2.3.5.4 *Biological effects of IL-10*

1.2.3.5.4.1 *Monocytes / Macrophages*

IL-10 acts on monocytes to inhibit cytokine, chemokine, and prostaglandin E2 production (Niiro, Otsuka and others, 1994; Niiro, Otsuka and others, 1995). It reduces expression of the LPS recognition and signalling molecules CD14 and Toll like receptor (TLR)-4, and inhibits antigen presentation, while enhancing phagocytosis, and expression of CD16; and CD64 (Muzio, Bosisio and others, 2000; Opal, Wherry, and Grint, 1998). In addition, IL-10 strongly inhibits synthesis at both the mRNA and protein levels of IL-1, -6, -8, -10, TNF- α , G-CSF, and GM-CSF, following activation by LPS or LPS+IFN- γ (de Waal, Abrams and others, 1991). The inhibitory effects of IL-10 on IL-1 and TNF- α production are central to its anti-inflammatory activity as these cytokines often act synergistically in inflammatory pathways and processes, and amplify responses by inducing secondary mediators such as chemokines, prostaglandins, and platelet activating factor (PAF).

IL-10 inhibits the differentiation of monocytes into dendritic cells, and promotes their maturation to macrophages with increased endocytic ability (Allavena, Piemonti and others, 1998). Despite the enhanced capacity of monocyte/macrophages to phagocytose opsonized particles, bacteria or fungi, IL-10 paradoxically reduces the ability of the cell to kill the ingested organisms by decreasing the generation of superoxide anion (O_2^-) and nitric oxide (NO) (Bogdan, Vodovotz, and Nathan, 1991; Cenci, Romani and others, 1993; Gazzinelli, Oswald and others, 1992; Moore, de Waal and others, 2001).

IL-10 reduces surface expression of MHC Class II antigens, CD54 (ICAM-1), CD80 and CD86, significantly impairing the T-cell activating capacity of monocytes (de Waal,

Abrams and others, 1991;de Waal, Haanen and others, 1991). It decreases both the constitutive and inducible levels of MHC Class II expression (Wanidworanun and Strober, 1993), and has been shown to inhibit the transport of mature peptide loaded MHC Class II molecules to the plasma membrane (Koppelman, Neefjes and others, 1997).

Whilst IL-10 inhibits the production of pro-inflammatory cytokines, it enhances the production of their natural antagonists. IL-10 enhances the production of IL-1ra and sTNFR -1 and -2 (Cassatella, Meda and others, 1994;Joyce and Steer, 1996;Moore, de Waal and others, 2001). Of note, human IL-10 displays auto-regulatory activity by inhibiting its own production by LPS-activated monocytes.

1.2.3.5.4.2 *T & B cells*

The cytokine-producing CD4⁺ helper T lymphocytes are classified into 2 functionally dichotomous subsets on the basis of the cytokines produced; Th1 and Th2. IL-10 strongly inhibits cytokine production of both subsets directly and indirectly. Indirectly IL-10 inhibits proliferation and cytokine production of CD4⁺T-cells via its down-regulatory affects on antigen presenting function (de Waal, Haanen and others, 1991;Fiorentino, Zlotnik and others, 1991b). In addition, IL-10 has been shown to directly inhibit cytokine production at both the protein and mRNA levels in Th1 cells (IL-2, -3, IFN-γ), Th2 cells (IL-4, -5), and NK cells (TNF-α, IFN-γ), when monocytes/macrophages are the antigen presenting cells (Fiorentino, Zlotnik and others, 1991b;Fiorentino, Zlotnik and others, 1991a;Moore, O'Garra and others, 1993;Vieira, Waal-Malefyt and others, 1991). This inhibition of cytokine production is not seen if dendritic cells are utilized as the APC (Fiorentino, Zlotnik and others, 1991a). In contrast, IL-10 has stimulatory effects on CD8⁺ T-cells, inducing their recruitment, cytotoxic activity, and proliferation (Groux, Bigler and others, 1998;Moore, de Waal and others, 2001).

Of particular interest is the more recent finding that activation of T-cells in the presence of IL-10 can induce a state of nonresponsiveness/anergy (Groux, Bigler and others, 1996;Groux, Bigler and others, 1998). This IL-10 mediated anergy appears to be related to the induction of a population of regulatory T-cells, distinct from Th1 and Th2 cells, which produce high levels of IL-10 (Cobbold and Waldmann, 1998;Groux,

O'Garra and others, 1997). To date these regulatory T-cells have been isolated under different conditions and exhibit a varying cytokine expression profile. Preliminary evidence suggests that the regulatory T-cells are induced by a subset of dendritic cells producing high levels of IL-10.

Interleukin-10 has no effect on expression of MHC Class II on human B cells. IL-10 enhances survival of normal B-cells by increasing expression of the anti-apoptotic protein bcl-2 (Itoh and Hirohata, 1995; Levy and Brouet, 1994). IL-10 is a potent co-stimulator of B cells resulting in increased B cell proliferation and differentiation/isotype switching, which can be further enhanced by the presence of IL-2 or IL-4 (Moore, de Waal and others, 2001; Rousset, Garcia and others, 1992). Combined, IL-10 and IL-4 have a synergistic effect on B cell proliferation.

1.2.4 Evidence for the role of these cytokines in morbidity following CPB

Each of the cytokines discussed above have been reported to play a role in the systemic inflammatory response seen following CPB, with circulating levels rising during or in the immediate post-operative period (Fig 1.8). What is currently known about relationship between surgery and systemic rise, and evidence from prior research that implicates each of these cytokines in the morbidity observed following CPB is outlined below.

1.2.4.1 Cardiopulmonary bypass and TNF- α

TNF- α is a potent pro-inflammatory cytokine that mediates much of the systemic inflammatory response seen in patients with sepsis (Blackwell and Christman, 1996; Pinsky, Vincent and others, 1993; Thijs and Hack, 1995). Therefore TNF was a prime candidate for mediating post perfusion syndrome.

TNF- α has been shown in studies involving both human and animal myocardium, to produce a dose dependent negative inotropic response within minutes of production (Cain, Meldrum and others, 1999). There are 3 reported potential mechanisms through which TNF- α can cause myocardial depression: (1) an immediate pathway mediated by

activation of the neural sphingomyelinase pathway (Oral, Dorn, and Mann, 1997), (2) a delayed pathway involving alterations in nitric oxide (NO) homeostasis, with a shift from the cardioprotective constitutively produced NO to increased expression of the inducible nitric oxide synthase (iNOS) (Finkel, 2002; Finkel, Oddis and others, 1992; Gulick, Chung and others, 1989; Kumar, Kumar, and Parrillo, 1999), and (3), activation and/or release of myocardial depressants such as IL-1 β and IL-18. The first and second of these mechanisms act by impeding Ca²⁺ release or desensitizing the myocytes to the effects of Ca²⁺ respectively, and predominantly affect systolic function (Cain, Meldrum and others, 1999; Goldhaber, Kim and others, 1996; Oral, Dorn, and Mann, 1997). TNF- α also induces structural damage to endothelial cells by rearrangement of actin filaments and loss of tight junctions resulting in capillary leak (Brett, Gerlach and others, 1989; Stephens, Ishizaka and others, 1988).

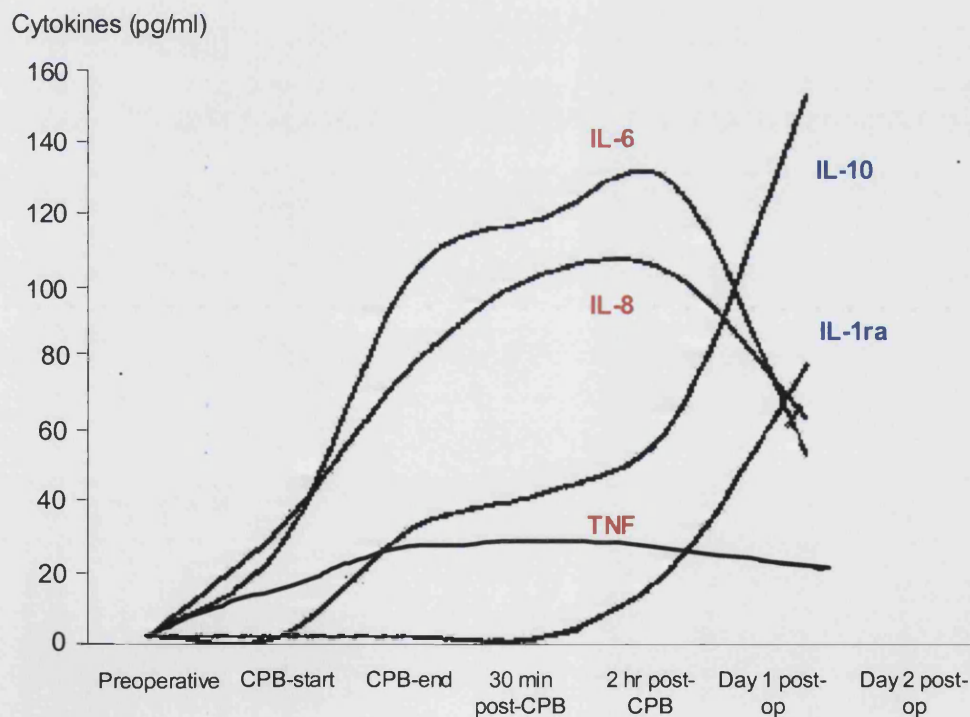


Figure 1.8 Hypothetical serum cytokine response in patients undergoing cardiac surgery with CPB (*Adapted from Brix-Christensen V and Chew M., 2001*)

While many studies have detected elevated levels of TNF- α following cardiac surgery, this is not always the case (McBride, Armstrong and others, 1996). Even when detected, concentrations are consistently lower than other circulating pro-inflammatory cytokines (Gilliland, Armstrong, and McMurray, 1998; Marano, Garulacan and others, 2000; Roth-Isigkeit, Hasselbach and others, 2001). The obvious limitation with interpreting plasma cytokine levels is that they do not necessarily reflect cytokine concentrations at the local tissue level. Whilst the main source for circulating TNF- α following cardiac surgery is probably monocytes and macrophages (Tracey and Cerami, 1993), the myocardium is also a recognised source (Wan, DeSmet and others, 1996). Levels of TNF- α have been shown to be significantly higher in coronary sinus blood compared to arterial blood. Thus low levels of TNF- α in plasma may not reflect the biological importance of TNF in outcome following CPB, however, the levels detected are markedly different from sepsis indicating potential differences in the aetiology of capillary leak, hypotension and end-organ dysfunction.

1.2.4.2 Cardiopulmonary Bypass and IL-6

Interleukin-6 has been consistently measured systemically following bypass in both paediatric and adult cardiac studies (Butler, Pathi and others, 1996; Defraigne, Pincemail and others, 2000; Roth-Isigkeit, Hasselbach and others, 2001; Rothenburger, Soeparwata and others, 2001; Wei, Kuukasjarvi and others, 2001). It is thought to be related to the extent of tissue injury, and has been used as a predictor of outcome (Hauser, Ben Ari and others, 1998; Oka, Murata and others, 1992). Plasma levels of IL-6 have been shown to correlate with, duration of aortic cross-clamping, circulatory arrest time and hypothermia (Hovels-Gurich, Vazquez-Jimenez and others, 2002; Steinberg, Kapelanski and others, 1993). Like TNF- α , IL-6 has been implicated in post bypass myocardial dysfunction via nitric-oxide dependant pathways (Finkel, Oddis and others, 1992; Hennein, Ebba and others, 1994). IL-6 is also thought to play a role in leukocyte-mediated injury to the myocardium by the induction of intercellular adhesion molecule-1 (ICAM-1) expression on cardiac myocytes (Sawa, Ichikawa and others, 1998; Youker, Smith and others, 1992). Measurable levels of IL-6 have been reported pre-operatively in children with congenital heart disease (Chew, Brandslund and others, 2001; Duval, Kavelaars and others, 1999).

1.2.4.3 Cardiopulmonary Bypass and IL-10

The stimulus for IL-10 production during cardiac surgery is uncertain. Adult studies report a rise in both IL-10 and pro-inflammatory mediators intra-operatively or within the first two hours of aortic cross clamp release (Dehoux, Philip and others, 1995; Tabardel, Duchateau and others, 1996). However similar studies in children have shown that the IL-10 response may precede any pro-inflammatory response (McBride, Armstrong and others, 1996; Seghaye, Duchateau and others, 1996).

It is unlikely that the IL-10 response arises as a direct result of contact of the blood with the bypass circuit, as neither IL-10, TNF- α , or IL-6 is detected in isolated CPB models (Gormley, Armstrong and others, 2003; McBride, Armstrong and others, 1996). There has been no consistent correlation between IL-10 levels and measures of surgical stress, although individuals have reported correlations between postoperative levels and duration of bypass or operative temperature (Sablutzki, Welters and others, 1997; Seghaye, Duchateau and others, 1996; Wan, LeClerc, and Vincent, 1997a). IL-10 has been reported to have cardioprotective effects. It inhibits neutrophil-endothelial interactions, and inhibits vascular smooth muscle proliferation (Hayward, Nossuli and others, 1997; Selzman, McIntyre, Jr. and others, 1998). Peri-operatively treatment with steroids significantly increases both IL-10 and IL-1ra production (Giomarelli, Scolletta and others, 2003; Kawamura, Inada and others, 1999; Rasmussen, Kelly, and Clausen, 2001; Seghaye, Duchateau and others, 1996; Tabardel, Duchateau and others, 1996; Tassani, Richter and others, 1999), suggesting a modulating effect of corticosteroids on IL-10 synthesis. Whether high levels of circulating IL-10 following CPB is protective or harmful is still not clear.

Yang et al (2000) attempted to address this issue in a model of myocardial ischemia/reperfusion using an IL-10 knockout mouse (Yang, Zingarelli, and Szabo, 2000). It was shown that IL-10 knock out mice had significantly greater reperfusion injury both histologically and biochemically. Serum TNF- α levels were much greater in the IL-10 knockout mice compared to the wild type mouse (>300 vs <100 pg/ml), suggesting that endogenously produced IL-10 serves to suppress the production of TNF- α following an ischemic/reperfusion event. Variation in IL-10 production may in part, explain why some studies fail to detect systemic overflow of TNF- α .

There is also significant interindividual variation in IL-10 secretion following cardiopulmonary bypass. Whilst genetics may play a role in this (discussed below), the presence of hypoxaemia pre-operatively has been associated with lower peri-operative IL-10 release (Hovels-Gurich, Schumacher and others, 2002).

1.2.4.4 Cardiopulmonary Bypass and other cytokines

A pronounced rise in plasma IL-1 receptor antagonist (IL-1ra) has been seen in both adults and children following CPB (Duval, Kavelaars and others, 1999). Consistent with *in vitro* findings where IL-10 leads to the production of IL-1ra, most studies show an IL-1ra response occurring late in CPB (Duval, Kavelaars and others, 1999;McBride, Armstrong and others, 1995;McBride, Armstrong and others, 1996). There is no data to suggest a direct role of IL-1ra on the heart or circulation, however it is recognised to play a significant role in limiting the inflammation and tissue damage caused by IL-1.

Raised plasma levels of IL-8 are consistently reported following cardiac surgery involving CPB (Defraigne, Pincemail and others, 2000;Finn, Naik and others, 1993;Rothenburger, Soeparwata and others, 2001). The plasma rise in IL-8 appears to coincide with the systemic rise in IL-6, beginning towards the end of theatre, peaking 1-3 hours post-op and remaining elevated at 24 hours (Defraigne, Pincemail and others, 2000;Finn, Naik and others, 1993;Rothenburger, Soeparwata and others, 2001). Plasma levels have been shown to correlate with duration of CPB and ischemic time, (Finn, Naik and others, 1993;Steinberg, Kapelanski and others, 1993). Both IL-6 and IL-8 have been shown to be produced by cardiac myocytes under conditions of ischemia-reperfusion, and levels have been found to reflect the extent of early myocardial damage (Dreyer, Phillips and others, 2000;Metinko, Kunkel and others, 1992;Pannitteri, Marino and others, 1997;Sawa, Ichikawa and others, 1998). IL-8 is a potent chemokine with neutrophil-stimulating properties. These properties have been postulated to have a causative role in the lung injury seen following CPB (Casey, 1993;Finn, Moat and others, 1993;Wan, LeClerc, and Vincent, 1997b). Interestingly the stimulus of CPB alone is sufficient to induce IL-8 secretion (Burns, Newburger and others, 1995;McBride, Armstrong and others, 1995).

1.2.5 Mechanisms to reduce the Inflammatory response to CPB

With the identification of the mediators of inflammation activated by CPB, and recognition of the mechanisms most likely to stimulate their release, strategies attempting to control the inflammatory response following surgery were developed. Techniques such as maintenance of haemodynamic stability, minimisation of exposure to CPB circuitry, pharmacological and immunological agents have all undergone clinical trials (recent review (Laffey, Boylan, and Cheng, 2002)). Hypothermia, haemofiltration and heparin bonded circuits are all currently utilised in an effort to decrease the inflammatory response to CPB and to minimise the occurrence of post-perfusion syndrome (Fig 1.9).

1.2.5.1 Minimisation of trauma

Advances in minimally invasive surgery such as laparoscopic and thoracoscopic procedures have prompted questions about whether the avoidance of a full median sternotomy would lessen the inflammatory response. There has been one non-randomised study which suggested shorter recovery to normal lifestyle, however at present operating times are considerably longer with minimal invasive techniques (Glower, Landolfo and others, 1998).

A crucial factor triggering the inflammatory response to CPB is the cellular trauma caused by exposure of blood to nonphysiological surfaces. Heparin bonding of the artificial surface was proposed as a means of reducing cellular activation. Heparin-bonded circuits have been shown to significantly reduce complement activity, granulocyte activation, platelet adhesion and circulating levels of TNF- α , IL-6 and IL-8 in children (Ozawa, Yoshihara and others, 2000). Their use however, has not been associated with improvement in outcome measures.

1.2.5.2 Leukocyte depletion

Circulating white blood cells play a central role in the inflammatory response to cardiac surgery. There is increasing evidence that leukocyte depletion, by means of leukocyte specific filters, may attenuate pulmonary and myocardial injury especially in patients with long bypass time (Johnson, Thomson and others, 1995; Morioka, Muraoka and others, 1996). A large scale clinical trial in adult CPB patients showed that

leukodepleting transfused blood significantly reduced the overall 60-day mortality primarily by reducing multiorgan failure (van de Watering, Hermans and others, 1998).

1.2.5.3 Removal of inflammatory mediators

Since the late 1990's, haemofiltration has been routinely used in children undergoing surgery requiring long bypass time, and most neonatal repairs. Haemofiltration has been associated with decreased systemic levels of IL-1, 6, 8, 10, TNF- α , and reduced complement activation (Bando, Vijay and others, 1998; Journois, 1998; Journois, Israel-Biet and others, 1996). Clinically there appears to be reduced postoperative bleeding, increased cardiac contractility, improved haemodynamic stability, and decreased length of stay (LOS) (Bando, Turrentine and others, 1998; Bando, Vijay and others, 1998; Davies, Nguyen and others, 1998; Journois, 1998; Journois, Israel-Biet and others, 1996). Initially it was believed that this reduction in post perfusion syndrome following haemofiltration was secondary to the filtering off of pro-inflammatory mediators, however it is probably due to the removal of excess free water.

1.2.5.4 Suppression of pro- and enhancement of anti-inflammatory mediators

Steroidal pre-treatment has been used on and off in cardiac surgery for 30 years, but its role in improving clinical outcome is still unproven. The main mechanism of action proposed is direct inhibition of NF- κ B, and hence reduced levels of pro-inflammatory cytokines, however this may be an over simplification of more complex cellular interactions (Lee and Burckart, 1998). In adult patients the use of peri-operative steroids has been shown to be associated with reduced systemic levels of pro-inflammatory cytokines and increased anti-inflammatory cytokines (particularly IL-10), however clinical benefits have not been consistently evident (Chaney, 2002; Giomarelli, Scolletta and others, 2003; Kawamura, Inada and others, 1999; Tabardel, Duchateau and others, 1996; Tassani, Richter and others, 1999; Wan, LeClerc, and Vincent, 1997b). Control trials using steroids in children undergoing CPB have not been associated with decreased levels of inflammatory mediators, and are not routinely used at the centre in which this work was undertaken.

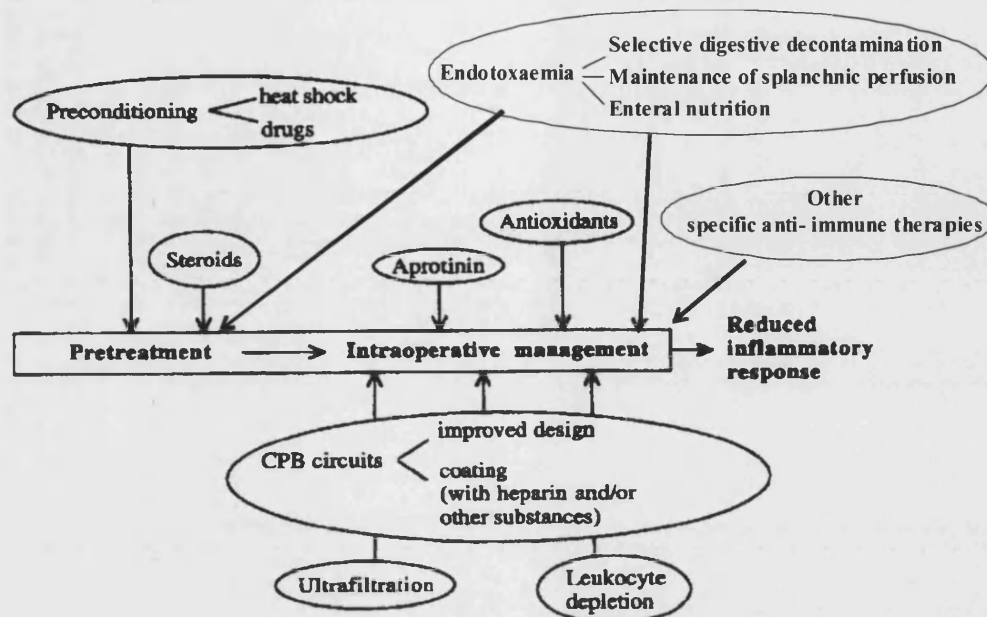


Figure 1.9 Therapeutic strategies to reduce inflammatory response to CPB (adapted from Wan S et al 1997)(Wan, LeClerc, and Vincent, 1997b).

Many effector proteins of the cytokine, complement, and haemostatic cascades are serine proteases → amplification of the inflammatory cascade. Inhibiting these proteases may limit inflammation to local tissues and reduce systemic overflow. The most commonly used serine protease inhibitor is aprotinin. Use of aprotinin at the end of cardiac surgery not only prevents excessive blood loss, but has pronounced anti-inflammatory effects (Levi, Cromheecke and others, 1999). Anti-inflammatory properties of aprotinin include:

- ⊕ increased IL-10 secretion (Hill, Diego and others, 1998)
- ⊕ attenuating platelet activation and maintenance of platelet function (Wachtfogel, Kucich and others, 1993)
- ⊕ decreased complement activation (Wachtfogel, Kucich and others, 1993)
- ⊕ decreased systemic release of IL-6, IL-8, and TNF- α (Harig, Feyrer and others, 1999; Soeparwata, Hartman and others, 1996)
- ⊕ inhibition of cytokine-induced iNOS induction (Hill and Robbins, 1997)

1.3 GENETICS AND INFLAMMATION

23

1.3.1 Candidate Genes Influencing the Intensity of the Inflammatory Response.

Allelic variations that exist stably in a population and have a frequency of at least one-percent or more are known as *polymorphisms*. The most frequent type of polymorphism in DNA is caused by single base-pair substitution (SNP). When the SNP is within a protein-coding region of a gene, the variant allele may lead to an amino acid substitution that functionally alters the resultant protein. Much more commonly SNPs occur in the nontranslated regions. These SNPs may still have biological effects. SNPs in the promoter region may alter binding affinity of transcription factors, such as NF- κ B, and therefore alter the rate of gene transcription and translation. SNPs within the 5'upstream region and 3'downstream region may alter stability of the transcribed messenger RNA (mRNA) or enhancer activity, therefore altering the efficiency of gene transcription and mRNA translation. SNPs within introns are less likely to have biological effects, although they may be linked with biologically active SNPs within other parts of the gene.

Three of the cytokines recognised to play an important role in the systemic inflammatory response and outcome following cardiopulmonary bypass have functional promoter polymorphisms. These are IL-6 -174, TNF- α -308, and IL-10 -1082, -819, and -592.

1.3.1.1 IL-6 -174 promoter polymorphism

IL-6 is rapidly cleared from the plasma, therefore the systemic concentrations of IL-6 are largely reflective of the level of gene expression (Castell, Geiger and others, 1988). Expression of IL-6 is tightly regulated by a number of transcription factors which bind to the IL-6 promoter region. In 1998 a single nucleotide polymorphism deriving from a G to C substitution was identified at position -174 of the human IL-6 promoter within a negative regulatory domain (Fishman, Faulds and others, 1998). The C allele is common in Caucasians (UK frequency 0.41, 95% CI 0.38-0.43) (Fishman, Faulds and others, 1998). In luciferase reporter vector assay, the -174 C construct exhibited both a decreased spontaneous and inducible expression of transcripts as compared to the -174 G construct (Morse, Olomolaiye and others, 1999). To further support this, the C allele

was associated with significantly lower plasma levels of IL-6 in healthy adults (Burzotta, Iacoviello and others, 2001; Fishman, Faulds and others, 1998).

These results lead to the concept that a certain IL-6 promoter genotype may be associated with a low or high IL-6 producer phenotype, which in turn may positively or negatively influence the outcome of IL-6 mediated pathology. A number of clinical situations have been studied describing their relationship with the -174 allele (Georges, Loukaci and others, 2001; Humphries, Luong and others, 2001; Jones, Brull and others, 2001; Marshall, Webb and others, 2002; Vickers, Green and others, 2002). Whilst in most cases the -174 G allele has been associated with high IL-6 production more recently data suggests that when the genotype is stressed (e.g. surgery), the -174 CC genotype results in significantly higher IL-6 production (Brull, Leeson and others, 2002; Brull, Montgomery and others, 2001).

1.3.1.2 TNF- α -308 promoter polymorphism

The gene coding for TNF- α is located within the MHC Class III region of chromosome 6, resulting in linkage disequilibrium between TNF- α alleles and certain MHC alleles. It is probably because of this that those diseases with an HLA association (e.g. rheumatoid arthritis, inflammatory bowel disease) frequently have an association with a TNF- α polymorphism. To date 11 polymorphisms have been reported in the TNF- α promoter region, some of which have been reported to affect either TNF- α production and/or TNF- α secretion.

Tumour necrosis factor- α is regulated both transcriptionally and post-transcriptionally. A G to A substitution at position -308 in the promoter region occurs with a frequency of about 30% in Caucasians (Mira, Cariou and others, 1999). The A allele has been reported to affect the ability of nuclear factors to bind to the -308 region (Kroeger, Carville, and Abraham, 1997), strongly enhancing transcriptional activation (Braun, Michel and others, 1996; Wilson, Symons and others, 1997) and has been associated with substantially increased (6-9 fold) *in vitro* gene transcription and protein synthesis (Bouma, Crusius and others, 1996; Heesen, Kunz and others, 2003; Louis, Franchimont and others, 1998; Morse, Olomolaiye and others, 1999; Wilson, Symons and others, 1997). The presence of the A allele has been associated with increased severity of

clinical disease in some, but not all studies (Allen, 1999; Mira, Cariou and others, 1999; Nadel, Newport and others, 1996; Stuber, Petersen and others, 1996). It is worth noting, the -308 polymorphism is actually only 307 base pairs upstream of the transcription site and was originally misnumbered (Ugialoro, Turbay and others, 1998))

1.3.1.2.1 *Clinical disease and the TNF- α polymorphism*

Just as TNF- α levels have been examined in a wide range of clinical pathologies, the finding of functional polymorphisms in the TNF- α promoter has quickly resulted in a number of studies examining their prevalence in diseases. The results of these studies have also been conflicting. Some authors believe that TNF- α genotype does influence clinical disease (Allen, 1999; Bouma, Crusius and others, 1996), whilst others conclude that TNF- α promoter polymorphisms exist solely because they are in linkage disequilibrium with HLA alleles, and are of no functional significance (Ugialoro, Turbay and others, 1998).

The -308 polymorphism has a strong association with the autoimmune haplotypes HLA-A₁, B₈, and DR₃ through linkage disequilibrium (Wilson, de Vries and others, 1993). However Bouma et al (1996) showed that secretion of TNF- α was elevated in individuals with the -308A allele whether they were DR₃⁺ or DR₃⁻, suggesting that linkage to HLA-DR₃ was not responsible for the difference in TNF- α production observed.

There have been four studies examining the association between the -308 polymorphism and outcome from sepsis, one of which was in children (Mira, Cariou and others, 1999; Nadel, Newport and others, 1996; Stuber, Petersen and others, 1996). Nadel et al reported a higher frequency of the TNF- α -308A allele in children who did not survive their admission to hospital with meningococcal disease (relative risk for -308 A/A homozygote 2.5; CI, 1.1-5.7) (Nadel, Newport and others, 1996). Ethnicity was not reported, nor was TNF- α levels measured in this study. Whilst these findings have not been reproduced, a multicentre adult study done a few years later showed increased prevalence of the TNF- α -308A allele in patients admitted to ICU with septic shock compared to healthy controls, and an association between -308A and death after controlling for age and severity of illness (Mira, Cariou and others, 1999). This study

however found no difference in serum TNF- α levels between groups stratified by genotype. In 2000, a study confirmed the association between the presence of the -308A allele and increased mortality among septic ICU patients, and for the first time reported higher circulating levels of TNF- α in non-survivors compared to survivors (Tang, Huang and others, 2000). The TNF- α -308 polymorphism has not been shown to play a role in the development of sepsis and outcome in a general surgical cohort (Stuber, Petersen and others, 1996).

There have been two studies examining the association between TNF- α -308 polymorphism and outcome following adult cardiac surgery and one examining the role of the polymorphism in heart transplant patients. Yende and colleagues studied 400 adult patients following coronary artery bypass grafting (CABG) and found that the presence of the more common -308G allele, in combination with the TGF- β +250G allele, was associated with prolonged mechanical ventilation independent of preoperative risk factors (Yende, Quasney and others, 2003). Ryan et al found no association between the -308 polymorphism and the development of lactic acidosis post-operatively (Ryan, Balding and others, 2002). In a study of 175 heart transplant recipients and 212 healthy controls, Densem and co-workers found an overrepresentation of the TNF- α -308A allele in patients with end-stage non-ischemic myocardial dysfunction (Densem, Hutchinson and others, 2002).

The -308A genotype has been associated with a number of clinical outcomes including bronchial hyper responsiveness in asthma (Li Kam Wa, Mansur and others, 1999), idiopathic dilated cardiomyopathy, celiac disease and severe outcomes in infectious diseases including meningococcal disease (mentioned above), cerebral malaria, leishmaniasis, and leprosy (McGuire, Hill and others, 1994; Nadel, Newport and others, 1996; Roy, McGuire and others, 1997). Levels of TNF- α production are also felt to be important in liver, cardiac and renal allograft rejection, where anti-TNF- α antibodies have been utilised in an attempt to reduce the rate of rejection. Some groups advocate screening all allograft recipients for the -308 polymorphism and tailoring the amount of immunosuppression according to the presence or absence of the polymorphism.

1.3.1.3 IL-10 promoter polymorphisms

Stimulation of human blood cultures with endotoxin reveals large inter-individual variation in IL-10 secretion (van der Linden, Huizinga and others, 1998), which has been shown in monozygotic twins to have a genetic component of over 70% (Westendorp, Langermans and others, 1997a; Westendorp, Langermans and others, 1997b). Levels of secreted IL-10 are directly proportional to levels of IL-10 mRNA. A series of bi-allelic polymorphisms in the promoter of the IL-10 gene, which regulate IL-10 transcription, have been described at positions -1082, -819, and -592 (Turner, Williams and others, 1997). Three major haplotypes have been reported – GCC, ACC, and ATA. The GCC haplotype is associated with the highest IL-10 levels (1.3 fold) using Con-A stimulated cells homozygous for the GCC haplotype (Eskdale and Gallagher, 1995; Turner, Williams and others, 1997; Westendorp, Langermans and others, 1997b). In western populations, GCC, ACC and ATA haplotype frequencies are 54.1%, 28.3% and 16.8% respectively. A rare GTA haplotype has a frequency of 0.6% (Eskdale and Gallagher, 1995). Recently it has become apparent that the GCC haplotype group should be subdivided into two groups ; GGCC and GACC due to a G/A polymorphism at –851 described by Alfonso et al and verified by Wood et al (Turner, Williams and others, 1997; Wood, Keen and others, 2001).

The promoter region for IL-10 contains putative binding sites for a number of transcription factors known to be involved in the regulation of other cytokines (Kube, Platzer and others, 1995; Kube, Rieth and others, 2001). However the precise transcription factors involved in IL-10 regulation in humans are still uncertain.

1.3.1.3.1 *Clinical disease and IL-10 polymorphisms*

Levels of IL-10 production are critical in immune regulation, controlling the balance between inflammatory and humoral responses, limiting and ultimately terminating inflammation. IL-10 has been suggested to play a role in peripheral tolerance and in protection against autoimmunity (Moore, de Waal and others, 2001). The concept that the presence or absence of a functional IL-10 promoter polymorphism could alter susceptibility to a chronic inflammatory or autoimmune disease has become much investigated. To date there has been no clear association between the prevalence or

severity of chronic hepatitis, multiple sclerosis, rheumatoid arthritis or systemic lupus disease and IL-10 genotype (Bathgate, Pravica and others, 2000; Eskdale, Gallagher and others, 1998; Eskdale, Wordsworth and others, 1997; Hajeer, Lazarus and others, 1998; Koch, Kastrati and others, 2001; Lazarus, Hajeer and others, 1997; Middleton, Taylor and others, 1998; Mok, Lanchbury and others, 1998). There has been only one study examining the association of an IL-10 polymorphism in sepsis (Lowe, Galley and others, 2003). This study of 67 critically ill patients admitted to ICU showed that the presence of the IL-10 -592C allele was associated with a higher IL-10 response following whole blood stimulation with endotoxin, but with decreased mortality. Recently an association of the IL-10 -1082G allele with lower circulating levels of IL-10 three hours following CPB has been shown, but there was no association between genotype and outcome measures (Galley, Lowe and others, 2003).

Because the nature of functional polymorphisms is to amplify an initial inflammatory response, promoter polymorphisms may have their greatest effect when the cytokine response to an inflammatory insult is unbalanced.

1.4 THE ROLE OF CYTOKINE BALANCE

Initially the model used to explain the clinical findings following cardiopulmonary bypass was one of persistent, uncontrolled inflammation, and the key mediators were thought to be pro-inflammatory. In 1996, Roger Bone's landmark editorial revised the model for inflammation and raised the possibility that an excessive anti-inflammatory response may also be harmful, and the concept of *immune balance* emerged (Bone, 1996). From this point, research into inflammation following an insult has trended away from identifying and blocking a single mediator of inflammation towards methods of quantitating the state of cytokine balance that exists, and targeting treatment towards restoring homeostasis (Fig 1.11).

1.4.1 The intricate balance that exists between TNF- α and IL-10

In contrast to TNF- α , IL-10 is constitutively produced at low levels by circulating monocytes and macrophages, and inhibits its own production via a negative feedback

loop. As mentioned before, high concentrations of IL-10 are a potent inhibitor of monocyte/macrophage production of TNF- α . IL-10 has been shown to inhibit both the proteolytic cleavage of the membrane bound TNF- α molecule and NF- κ B activation during TNF- α transcription (Bogdan, Paik and others, 1992). In addition IL-10 increases the synthesis and release of sTNFR-2 at a transcriptional level (Dickensheets, Freeman and others, 1997). Figure 1.10 illustrates the effect of IL-10 on the immune balance between TNF- α and sTNFR-2. As the dose of IL-10 increases, TNF- α levels decrease, whilst levels of the inhibitory sTNF-R increase.

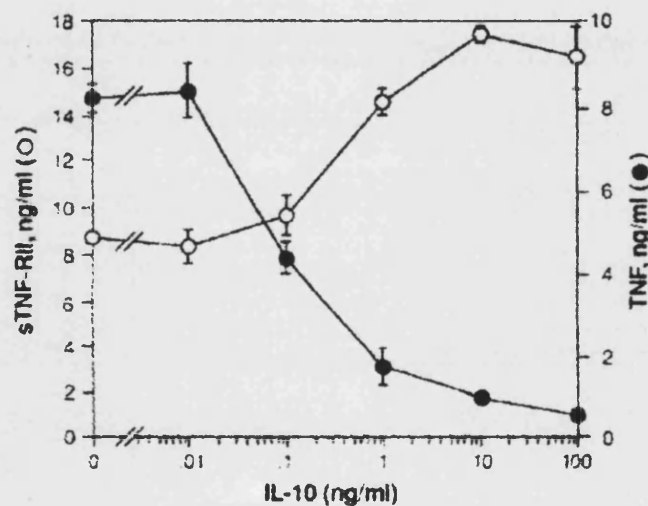


Figure 1.10 The dose related effect of IL-10 on production of TNF- α and sTNFR-2 by LPS stimulated monocytes (adapted from Wan S et al 1998).

In healthy individuals, although circulating levels of TNF- α are negligible, TNF- α mRNA levels in monocytes increase strikingly within 15-30 minutes of stimuli such as LPS. In contrast, LPS-induced IL-10 expression by monocytes *in vitro* occurs several hours after activation (de Waal, Abrams and others, 1991). TNF- α increases monocyte/macrophage production of IL-10 by increasing mRNA, however a second stimulus appears to be required before this increased mRNA is translated into increased protein levels.

TNF- α acts through a positive feedback mechanism to increase its own production (Philip and Epstein, 1986). Thus, in the presence of a functional promoter polymorphism, a small initial increase in TNF- α production secondary to an inflammatory insult may be magnified considerably during an *in vivo* immune response with the potential to have a significant effect on outcome.

1.4.2 Cytokine balance and sepsis

The concept of cytokine balance has been most intensively studied in the field of sepsis. In humans, both TNF- α and IL-10 are produced during septicaemia and septic shock, and serum levels have been shown to correlate with the clinical grading of the inflammatory response (Sherry, Cue and others, 1996; Tracey and Cerami, 1993; van der, de Waal and others, 1997). Research has shown both high and low circulating levels of either IL-10 or TNF- α to be associated with poor clinical outcome (van der, de Waal and others, 1997).

Repeatedly, high levels of IL-10 have been shown in experimental models to be protective against lipopolysaccharide (LPS) induced septic shock (Gerard, Bruyns and others, 1993; Howard, Muchamuel and others, 1993). The administration of IL-10 improves survival in animal models of endotoxaemia, and reduces systemic symptoms (e.g. fever, tachycardia), neutrophil responses, and cytokine production in human volunteers given endotoxin (Marchant, Bruyns and others, 1994; Pajkrt, Camoglio and others, 1997). Mice that have genetic deletions of the IL-10 gene are more susceptible to endotoxic shock than normal mice (Dai, Kohler, and Brombacher, 1997). Equally so, a physiologically inadequate IL-10 response after systemic injury has been shown to have detrimental consequences: - low lung concentration of IL-10 in patients with acute lung injury is associated with a greater risk of acute respiratory distress syndrome (ARDS) (Donnelly, Strieter and others, 1996). An inadequate IL-10 response to systemic injury or endotoxin challenge is associated with excess pro-inflammation, poor outcome, and increased mortality.

Yet in contrast, it has been reported that IL-10 levels are highest in critically ill patients with sepsis, multi-organ failure and in non-survivors (Doughty, Carcillo and others,

1998). Whilst this may just be indicative of the severity of the underlying disease in these patients, it may also represent the opposite end of a continuous spectrum. Patients who preferentially express high levels of IL-10 and reduced levels of TNF- α when challenged, are more likely to die from meningococemia and a variety of other community acquired infections (Pajkrt, Camoglio and others, 1997; van Dissel, van Langevelde and others, 1998; Westendorp, Langermans and others, 1997a). It appears that there is evidence to support that too little or too much of either pro-inflammation or anti-inflammation in the systemic inflammatory response can be associated with poor outcome (Howard, Muchamuel and others, 1993).

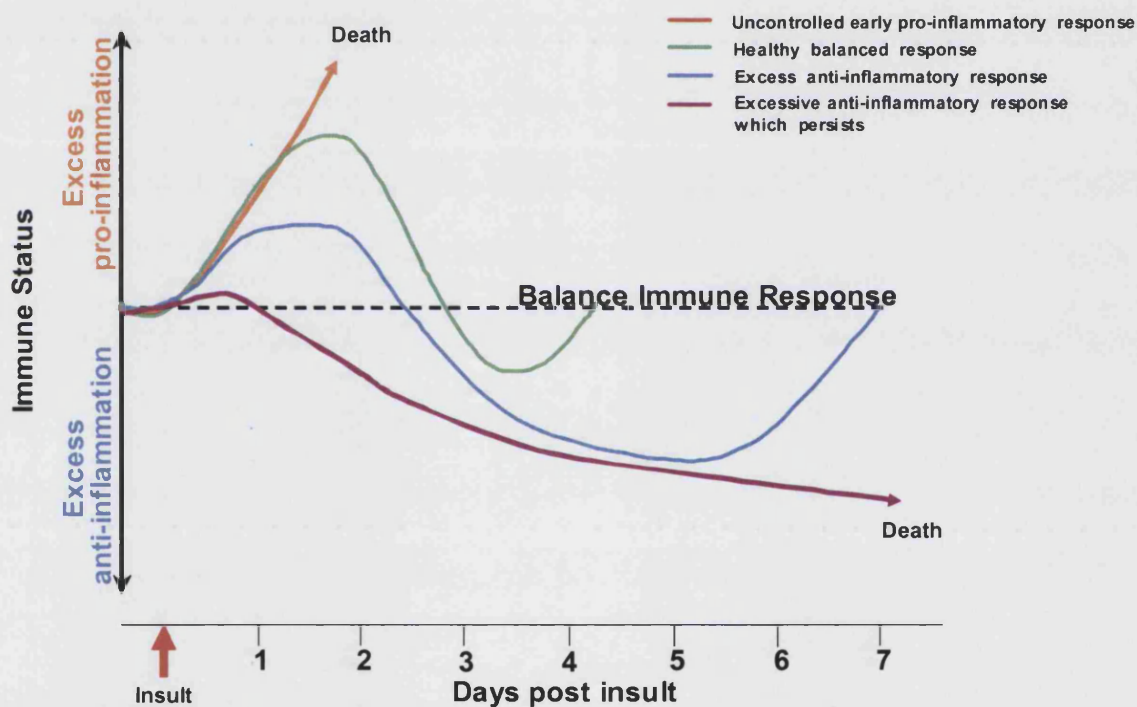


Figure 1.11 Hypothetical responses of a patient following an inflammatory insult. A balanced immune response involves an initial pro-inflammatory response with a rapid counter-balancing anti-inflammatory response, resulting in the systemic levels of cytokines returning rapidly to normal. An excessive early pro-inflammatory response to the insult results in SIRS, multi-organ failure, and high risk of early death. Prolonged or extreme anti-inflammatory response to the primary insult places the patient at risk of increased susceptibility to secondary infection, multi-organ failure, and late death.

The same model of inflammation (Sepsis-SIRS-CARS) probably holds true for surgery and trauma, including cardiopulmonary bypass. The most likely and important outcome

factor after good surgical repair may be the inter-relationship between the pro-inflammatory and anti-inflammatory mediators produced and immune balance.

1.4.3 Quantitating cytokine balance

The concept of balance and imbalance in a complex, apparently chaotic system of inter-related mediators is very attractive. However the problem arises of how to best assess balance. Attempting to assess balance based simply on circulating cytokine levels raises the dilemma discussed in section 1.2.3.1:- are systemic levels necessarily reflective of local tissue levels? Systemic levels may greatly underestimate the biological impact a cytokine is having locally (Wan, DeSmet and others, 1996). In addition, the complex interplay between cytokines at any single moment, their ubiquitous distribution, and short half-life, means that it is highly unlikely that a simple ratio between 2 circulating cytokines (e.g. IL-6:IL-10) at a single time point would adequately assess the inflammatory system (Hovels-Gurich, Schumacher and others, 2002).

The biological features of cytokines and their highly complex, and still poorly understood, interactions with each other, make them unlikely candidates for quantitating the resultant effect of an inflammatory response. Two alternative methods have emerged:

- monocyte hypo-responsiveness to lipopolysaccharide / endotoxin stimulation
- reduced surface expression of Major Histocompatibility Complex (MHC) Class II on monocytes

1.5 MONOCYTES AND SURFACE EXPRESSION OF MHC CLASS II

1.5.1 Background

Monocytes are immune cells derived from the bone marrow through the myeloid lineage. They constitute 5-8% of total circulating leukocytes in paediatric whole blood ($2 - 8 \times 10^5$ monocytes/ml of blood). The monocyte measures 16-20 μ m in diameter with

a deeply indented or U-shaped nucleus (Figure 1.12). Monocytes circulate in blood for approximately a day ($t_{1/2} = 17$ hours), before leaving and entering the tissues where they mature and differentiate into macrophages, with a life-span of months to years.

Monocyte production and release from the bone marrow is increased during an inflammatory response. Circulating monocytes have pinocytic and phagocytic activity and express receptors for immunoglobulin and complement. They are the major antigen presenting cells in the circulation, playing a central role in innate immunity by processing and presenting antigen in association with major histocompatibility complexes Class I and II. In addition, monocytes are able to traffic to sites of infection by utilising their migratory and chemotactic functions.



Figure 1.12 Normal peripheral blood monocyte (*courtesy Haematology Department, Great Ormond Street Hospital*).

1.5.2 Major Histocompatibility Complex (MHC) Class II structure and function

The major histocompatibility complex (MHC) describes a collection of genes sited on the short arm of Chromosome 6 that code for molecules involved in immune function.

The MHC Class II molecules are responsible for presenting peptides derived from external pathogens to $CD4^+$ T cells. In man there are 3 classical MHC Class II molecules known as Human Leukocyte Antigens or HLA - DR, DQ and DP. In addition to these structures, the MHC region encodes for so-called nonclassical molecules; HLA-DM and HLA-DO. These molecules do not reside on the cell surface and are important in modulating the binding of peptides to classical MHC molecules and cellular trafficking.

MHC (or HLA) molecules are cell-surface glycoproteins with a peptide-binding groove. MHC class II molecules (MHC Class II) in humans consist of 2 chains - α and β , which span the cell membrane. Each chain has 2 extracellular domains, α_1 and α_2 , and β_1 and β_2 respectively. In MHC Class II, the distal α_1 and β_1 domains fold together to form a single peptide-binding site composed of two anti-parallel α -helical loops supported by a platform of eight antiparallel β strands (Fig 1.13).

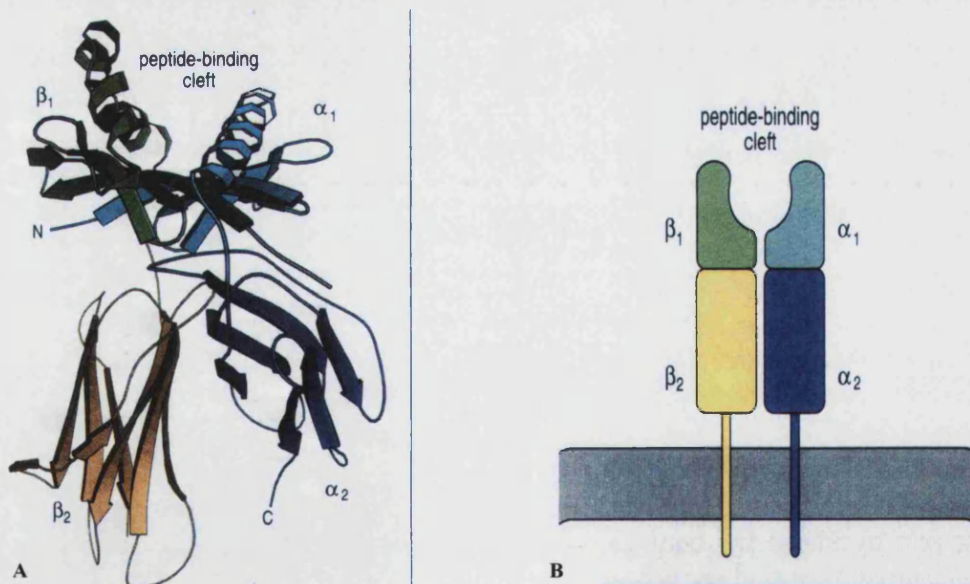


Figure 1.13 Structure of MHC Class II molecules.

The MHC Class II molecule is composed of 2 transmembrane glycoproteins chains (α and β). Figure 1A is a ribbon diagram of the structure, illustrating the α_1 and β_1 domains, which form the peptide binding groove. Figure 1B is a schematic diagram showing the peptide binding groove and the α_2 and β_2 domains, which span the cell membrane. (Adapted from Janeway CA et al. 4th edition 1999).

There are several characteristics of the MHC Class II binding groove that enable binding of a wide range of peptides:

- the α_1 and β_1 domains feature a high level of polymorphism
- the ends of the binding groove are open allowing peptides to bind in an extended conformation
- the peptide is held by a series of hydrogen bonds between the peptide backbone and conserved amino acid side chains lining the groove. Since the bonds do not involve peptide side chains, they confer sequence-independent binding.

Combined, these features help to explain how MHC Class II molecules can bind multiple peptides with high affinity and low specificity. In contrast the peptide binding site of the nonclassical Class II molecule HLA-DM is an almost fully closed groove, with the α_1 and β_1 domain in contact over the first and last thirds of their length.

MHC Class II molecules are generated in the endoplasmic reticulum (ER). In order to prevent them binding prematurely to ER-resident peptides, each newly synthesised molecule is associated with a protein known as the MHC Class-II associated invariant chain (Ii). Each invariant chain can occupy the binding groove of three MHC Class II molecules (Fig 1.14). The grooves of the MHC Class II molecules are occupied by a section of the Ii chain called CLIP. In the absence of invariant chains it appears that MHC Class II molecules are retained in the ER as complexes with misfolded proteins.



Figure 1.14 Invariant chain occupies 3 newly synthesised HLA-DR molecules. The invariant chain (in green) occupies the protein binding groove of 3 newly synthesised HLA-DR molecules (in yellow) in the ER to prevent binding of other proteins synthesised by the ER. (*Adapted from Janeway CA et al. 4th edition 1999*).

The main function of the invariant chain seems to be as a chaperone to ensure correct folding and delivery of MHC Class II molecules from the ER to the endosomal compartment where peptide loading can occur. The chain contains a di-leucine targeting signal in its cytoplasmic tail which helps to divert the folded complex from the default secretory pathway to lysosomal-like vesicles called MIIC (MHC Class II compartment), where peptide is loaded. The MHC Class II:invariant chain complexes are then exposed to an acidic, proteolytic environment in which the invariant chain is cleaved by proteases such as cathepsin L and S leaving just the CLIP fragment occupying the binding groove (Fig 1.15). Cathepsin S inhibition *in vitro* and cathepsin S deficient mice results in a profound reduction in MHC Class II surface expression (Nakagawa, Brissette and others, 1999).

Preferential exchange of CLIP for antigenic peptides is mediated by HLA-DM. With a binding groove effectively sealed, the structure of HLA-DM makes it highly unlikely to bind peptides. Most of the DM molecules reside in the MIIC vesicles where it binds transiently to MHC Class II:CLIP and stabilizes an intermediate state where CLIP can be released and peptide loaded. Once antigenic peptide has been stably bound, it has been proposed that DM loses its affinity for MHC Class II (Ting and Trowsdale, 2002).

The role of the other nonclassical molecule, HLA-DO, is less certain. Like DM it resides in the MIIC vesicles and appears to counterbalance the effect of DM. A narrow acidic pH range of the MHC Class II compartment has been described as essential for antigenic peptide loading and thus for surface expression of MHC Class II molecules (Ullrich, Doring and others, 1997). At a pH of 6 HLA-DO appears to block peptide exchange, however at lower pH's (pH=5) the acidic environment favours DM-mediated peptide exchange.

MHC Class II molecules in uninfected cells bind peptides derived from self-proteins. In addition, immature unloaded MHC Class II molecules, together with the invariant chain, have been shown to be directly transported to the cell surface with subsequent re-endocytosis and peptide loading (Roche, Teletski and others, 1993). Empty MHC Class II molecules that do not bind peptide after cleavage of the invariant chain are unstable,

and rapidly degraded in the acidic pH of the MIIC vesicles. At any time it appears that there is excessive production of MHC Class II molecules; therefore should infection occur, peptides generated from the pathogen are rapidly associated with empty MHC Class II molecules. MHC molecules must be able to bind, not only a wide variety of peptides, but also bind the peptide with high affinity so that peptide exchange at the cell surface does not occur.

Additional genes have also been identified in the class II region, some of which encode for proteins involved in antigen presentation.

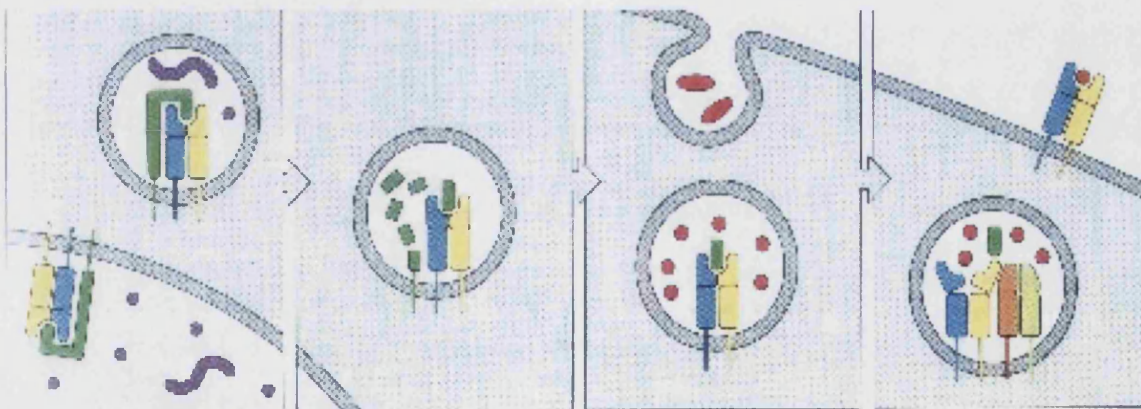


Figure 1.15 The transition of MHC Class II from the endoplasmic reticulum, through protein loading, to antigen presentation on the surface of the monocyte. The far left box shows newly synthesised MHC Class II in a vesicle. The protein binding groove is not available to bind peptides or misfolded proteins present in the vesicle because of the invariant chain. The second box shows cleavage of the invariant chain in the acidic environment. The binding groove still being occupied by the short peptide fragment known as CLIP. The vesicle then fuses with an endosome containing peptides from a foreign antigen. The final box shows removal of the CLIP protein and binding of the antigen. This is assisted by the presence of the HLA-DM molecule. (Adapted from Janeway CA et al. 4th edition 1999).

1.5.3 MHC Class II regulation

Expression of MHC Class II genes is tightly regulated. In contrast to the ubiquitous expression of Class I molecules, MHC Class II molecules are expressed on only a small number of specialised cells. Constitutive expression of MHC Class II is largely restricted to cells that serve as antigen presenting cells for CD4⁺ T-cells, such as B-lymphocytes, dendritic cells, monocytes and macrophages. Expression on other cell types can be induced by IFN- γ .

Many agents are recognised to modulate MHC Class II expression depending on cell type. Basal expression can vary according to the developmental stage of the cell – early B-lymphocytes are MHC negative, but expression can be induced by IL-4. Immune and neuroendocrine mediators can further modulate constitutive expression. For example IL-4, IL-10, and IL-13 all increase MHC Class II expression in B-lymphocytes, whilst prostaglandins and glucocorticoids decrease expression (Defrance, Carayon and others, 1994; Erb, Holtschke and others, 1994; Glimcher and Kara, 1992; Go, Castle and others, 1990). GM-CSF is a powerful inducer in dendritic cells (Sallusto and Lanzavecchia, 1994). IFN- γ increases levels of expression on existing cells (monocyte – macrophage lineage, endothelial cells) and can induce expression on certain cell types that do not normally express MHC Class II (epithelial cells, fibroblasts, and muscle cells). The same modulator can have opposite effects in different target cells. For example, IL-10 inhibits basal and IFN- γ induced MHC Class II expression on monocytes (de Waal, Abrams and others, 1991) but increases expression on B lymphocytes (Go, Castle and others, 1990). MHC Class II expression may also be mediated by direct cell-to-cell contact. CD40-CD40L and CD5-CD72 interactions increase MHC Class II expression in B-lymphocytes and dendritic cells (Sallusto and Lanzavecchia, 1994), whilst MHC Class II expression on endothelial cells can be induced by contact with Natural Killer (NK) cells (Watson, Petzelbauer and others, 1995). Whilst many of these effects occur at a transcription/translation level, TNF- α , LPS, GM-CSF and IL-10 have also been shown to modulate cell surface MHC Class II expression at a post-translational level by altering rates of exocytosis and recycling (Cella, Engering and others, 1997; Koppelman, Neefjes and others, 1997; Pierre, Turley and others, 1997).

Expression of MHC Class II genes is regulated primarily at the level of transcription. The main control element regulating transcription is the highly conserved promoter proximal region situated upstream of the transcription initiation site. Additional promoter distal regulatory elements have been described, but these appear to play a lesser role (Glimcher and Kara, 1992).

MHC Class II molecules are critical for the generation and maintenance of an immune response. Congenital absence of MHC Class II surface expression results in a severe immunodeficiency, whilst over expression is often seen in the setting of autoimmune diseases.

1.5.4 MHC Class II deficiency (Bare Lymphocyte Syndrome)

Bare lymphocyte syndrome (BLS) is a heterogeneous group of genetic disorders characterised by a lack of MHC Class II expression on both the thymus and antigen presenting cells. The disease is autosomal recessive and associated with extreme susceptibility to viral, bacterial, fungal, and protozoal infections. Cells from a BLS patient typically lack constitutive and inducible Class II, have reduced CD4⁺ cell numbers, and severely impaired T-cell activation. Understanding the precise regulation of MHC Class II expression and the involvement of promoters and transcription factors has largely been achieved through the study of the heterogeneous group of defects present in BLS patients.

This syndrome is not caused by mutations in the MHC genes themselves, but by a specific defect in a transcription factor that is necessary for MHC Class II expression. Bare lymphocyte syndrome is a disease of gene regulation. One gene defect involves the MHC Class II transactivator (CIITA).

1.5.5 CIITA: the master control factor for MHC class II expression

CIITA does not bind DNA directly, but mediates its function as a transcription factor through interaction with other proteins. Levels of CIITA expression precisely parallel that of MHC Class II synthesis both *in vitro* and *in vivo* (Sims and Halloran, 1999; Ting and Trowsdale, 2002). CIITA is constitutively expressed in the same cell types that constitutively express MHC Class II, and is not expressed in those cells that do not have

MHC Class II. CIITA transcription is increased by IFN- γ , endotoxin, and IL-4, and is decreased by IL-10, TGF- β , nitric oxide and IFN- β *in-vitro* (Harton and Ting, 2000; Reith and Mach, 2001). When induced, CIITA expression precedes MHC Class II mRNA induction by several hours. Over expression of recombinant CIITA by a factor of 5-10 above wild type levels in transfected cells is accompanied by a corresponding increase in MHC Class II expression (Mach, Steimle and others, 1996), whilst fibroblasts from CIITA-deficient Bare Lymphocyte Syndrome patients cannot be induced by IFN- γ to express MHC Class II molecules. Constitutive expression of recombinant CIITA renders MHC Class II negative cell lines MHC Class II positive in the absence of IFN- γ . Therefore, it appears that the level of CIITA protein determines MHC Class II promoter activity.

CIITA itself is regulated primarily at the transcription level, with most regulators acting through promoters (Fig 1.16). There are 4 recognised promoters (P1-4). Each promoter appears to lead to the expression of a different isoform of CIITA. Some isoforms have been shown to be more efficient at activating the Class II promoters than others, and their activation may explain the differing surface expression of Class II amongst different cell types. In some cases such as IFN- γ the promoter is known, but in other cases such as IL-10 the promoter affected is less clear.

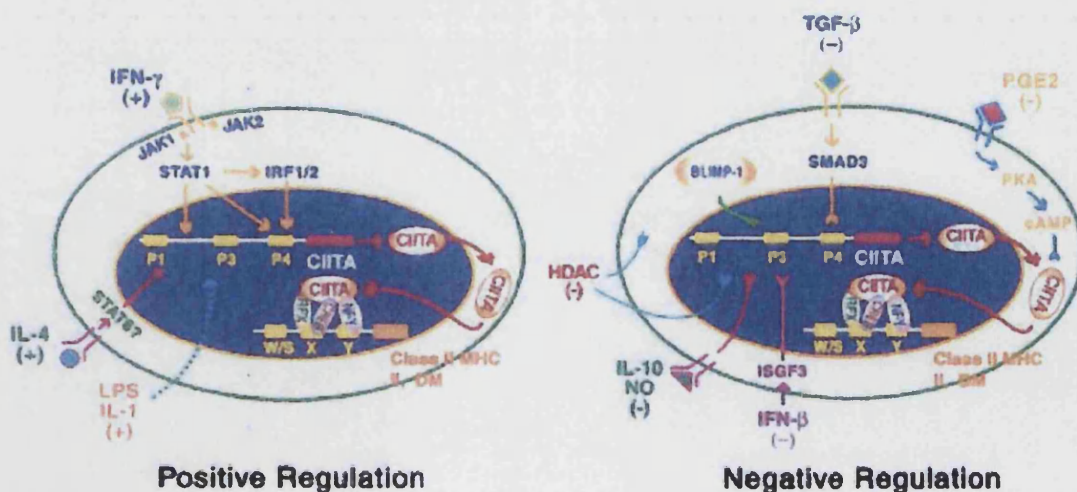


Figure 1.16 Positive and negative regulation of CIITA.

Regulatory processes of MHC Class II typically target CIITA transcription or protein. Positive regulators include IFN- γ , LPS, IL-4 and IL-1. IFN- γ acts on CIITA promoters P3 and P4. Negative regulators include IL-10, TGF- β , NO, and IFN- β . TGF- β acts to suppress P4, however the mechanism of action for IL-10 is not known. (Adapted from Ting JP and Trowsdale J 2002).

Once CIITA has been produced it interacts with a large number of DNA-binding transcription factors across the MHC Class II promoter region to form an active and more stable transcriptosome (Fig 1.17). Whilst the presence of CIITA is important, it is not essential. One transcription factor, regulatory factor X (RFX), appears to be crucial for the transcriptosome to access the Class II promoters (Kara and Glimcher, 1991a; Kara and Glimcher, 1991b). In RFX-defective cell lines, the Class II promoter remains bare even in the presence of IFN- γ (Brickey, Wright and others, 1999). In the absence of CIITA, the other transcription factors appear to bind weakly to the Class II promoters in cells that constitutively express MHC Class II. However, the presence of CIITA ensures proper histone acetylation of the transcriptosome making it both more stable and more active (Beresford and Boss, 2001; Harton, Zika, and Ting, 2001; Ting and Trowsdale, 2002). CIITA $-/-$ mice are largely devoid of MHC Class II, although there is some residual expression (Landmann, Muhlethaler-Mottet and others, 2001; Williams, Malin and others, 1998). Despite this however, both RFX $-/-$ and CIITA $-/-$ mice show severe immunodeficiency and CD4⁺ T-cell defects that replicate the findings in humans with BLS (Ting and Trowsdale, 2002).

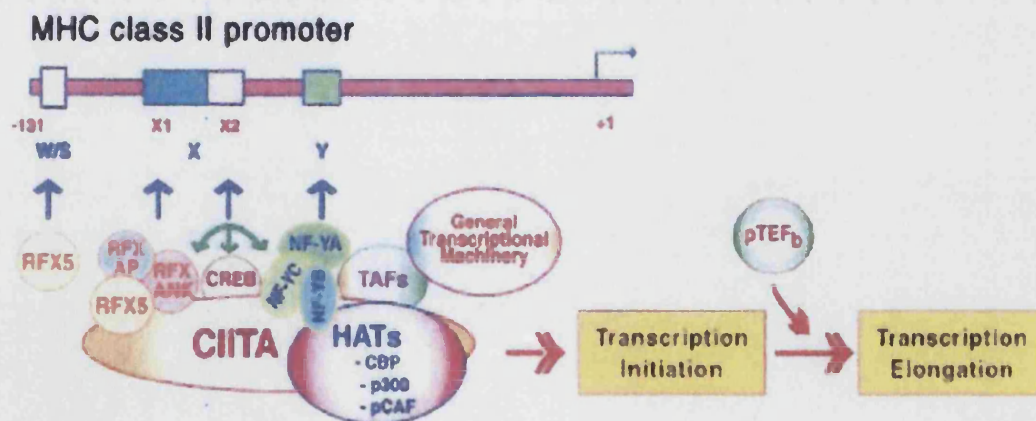


Figure 1.17 Schematic diagram of MHC Class II promoter and transcriptosome. A number of DNA binding factors (RFX, NF-Y, and CREB) along with the co activator CIITA bind to the MHC promoter activating transcription of the Class II gene. (Adapted from Ting JP and Trowsdale J 2002).

Polymorphisms within the CIITA promoters have recently been identified, but their significance is unclear. In theory they could result in a difference in the responsiveness of the cell to the local environment, with altered CIITA and MHC Class II induction, and variable T-cell response to infection (Rasmussen, Kelly, and Clausen, 2001).

1.5.6 IL-10 and MHC Class II expression

IL-10 has been shown to decrease both the constitutive and IFN- γ induced expression of MHC Class II on monocytes *ex vivo* in a dose dependant manner (de Waal, Haanen and others, 1991). In the presence of IL-10, monocytes had reduced capacity to present antigen and induce CD4⁺ T cell proliferation (de Waal, Haanen and others, 1991). In 1997 Koppelman et al demonstrated that IL-10 affects antigen presentation by inhibiting the post-translational processes of MHC Class II exocytosis and recycling (Koppelman, Neefjes and others, 1997). Subsequently IL-10 has been shown to have several post-translational biological effects. It has been shown to modify the pH of the MIIC vesicles, block the activity of cathepsin enzymes required for efficient MHC Class II peptide loading, and increase the activity of cystatin C an inhibitor of cathepsin (Fiebiger, Meraner and others, 2001; Longoni, Piemonti and others, 1998; Morel, Quarantino and others, 1997; Pierre and Mellman, 1998). However the signalling molecules activated by ligation of IL-10 to its receptor, that are responsible for these post-translational effects remain to be identified.

The idea that the reduction in MHC Class II expression on monocytes is occurring at a post-translational stage was support by a recent study in septic adult patient (Fumeaux and Pugin, 2002). Whilst having significantly decreased surface expression of MHC Class II, monocytes from septic patients had no significant difference in mRNA levels of HLA-DR and related molecules critical for DR expression compared to normal controls. This clinical study could not however address the question of whether the reduced surface expression was due to the sepsis or whether the MHC Class II decrease preceded the infection, and therefore represents a 'risk factor' for the development of sepsis.

1.6 MONOCYTE ‘*DEACTIVATION*’ AND *IMMUNE PARESIS*

1.6.1 Endotoxin (LPS) tolerance

In order to discuss monocyte *deactivation* and *immune paresis*, it is necessary to first describe a state termed *endotoxin tolerance*. Endotoxin tolerance was first described in the late 1960’s, when it was observed that animals given a low dose of LPS had a markedly reduced mortality when re-challenged with a higher, normally lethal dose of LPS (Brooke, 1965; Greisman, Young, and Carozza, Jr., 1969). Re-exposure of animal or cell lines to LPS after a previous challenge was associated with diminished TNF- α secretion. This impaired TNF- α secretion was thought to be an adaptive response, designed to limit pro-inflammation, and at least in part, explain the decreased mortality observed in tolerant animals.

An altered immune response similar to that seen with endotoxin tolerance has been shown to occur in critically ill patients, particularly those with sepsis. *Ex vivo* LPS challenges of both whole blood and monocytes isolated from septic patients has been associated with a reduction in TNF- α secretion (Cross, 2002; de, I, Zanetti and others, 2001; Kawasaki, Ogata and others, 2001; Ogata, Okamoto and others, 2000; Randow, Syrbe and others, 1995; West and Heagy, 2002; Yadavalli, Auletta and others, 2001). In addition, plasma from septic patients has been shown to induce hypo-responsiveness in control monocytes (Brandtzaeg, Osnes and others, 1996). Parenteral treatment with the pro-inflammatory cytokines TNF- α or IL-1 can induce an identical state.

The mechanism behind endotoxin tolerance are not yet understood but is thought to be an anti-inflammatory mediated effect. A common feature appears to be the presence of systemic inflammation. The state of endotoxin tolerance has been shown to occur in adult trauma and surgical patients, and this altered host defence is considered a risk factor for the subsequent development of septic complications (Kawasaki, Ogata and others, 2001; Keel, Schrengenberger and others, 1996; Lemaire, van der and others, 1998). One cell which appears to be central in the development of endotoxin tolerance is the monocyte.

1.6.2 Monocyte *deactivation*

The term of monocyte ‘*deactivation*’ has been coined to describe a decrease in surface HLA-DR expression and a reduced capacity of monocytes to secrete pro-inflammatory cytokines on subsequent LPS stimulation (Brandtzaeg, Osnes and others, 1996). The major candidate for the induction of monocyte *deactivation* is IL-10. Plasma concentration of the anti-inflammatory cytokine IL-10 has been shown to be greatest in the serum of patients who demonstrate monocyte *deactivation*, and removal of IL-10 in *in-vitro* cultures using a monoclonal antibody partially restores the capacity of monocytes to respond to LPS (Brandtzaeg, Osnes and others, 1996).

1.6.3 Monocyte *deactivation* and Immune Paresis

MHC Class II expression, monocyte responsiveness and circulating IL-10 levels appear to be intricately related. Low levels of monocyte MHC Class II expression, monocyte hypo-responsiveness and elevated levels of plasma IL-10 have all individually been shown to correlate with poor clinical outcome (Brandtzaeg, Osnes and others, 1996; Cheadle, Hershman and others, 1991; Ditschkowski, Kreuzfelder and others, 1999; Haveman, Kobold and others, 1999; Sachse, Prigge and others, 1999; Westendorp, Langermans and others, 1997a). Monocyte MHC Class II expression is, in part, under the regulation of cytokines in the micro-cellular environment. Reduced surface expression on circulating monocytes may reflect immune dysregulation, with an excessive anti-inflammatory response. *In vitro* experiments have provided the final link by showing that monocytes expressing low levels of HLA-DR not only have impaired antigen presentation, but secrete markedly reduced amounts of pro-inflammatory cytokines (de Waal, Haanen and others, 1991; Piani, Hossle and others, 2000), suggesting that MHC Class II may have a direct role in mediating the cellular response to endotoxin. Thus, prolonged or extreme release of anti-inflammatory mediators following an inflammatory insult may contribute to impaired monocyte function by down regulating MHC Class II expression, impairing antigen presentation, and decreasing pro-inflammatory cytokine release. This is termed a state of '*immune paresis*'.

1.7 HYPOTHESIS AND AIMS OF THIS THESIS

Placing all these facts together it is possible formulate a hypothesis. An unbalanced inflammatory response where IL-10 and other anti-inflammatory mediators predominate over TNF- α and other pro-inflammatory mediators, results in reduced monocyte MHC Class II expression and a state of monocyte '*deactivation*'. Under these conditions a second inflammatory insult would be poorly tolerated (*immune paresis*) with reduced capacity to mount an inflammatory response, placing the patient at risk of severe sepsis.

The aims of this thesis are:

1. To determine if monocyte surface MHC Class II expression is affected by cardiopulmonary bypass in children
2. To determine if alteration in MHC Class II expression is associated with an increased risk to the subsequent development of sepsis / systemic inflammatory response syndrome.
3. To examine the systemic pro- and anti-inflammatory cytokine response to cardiopulmonary bypass and examine its relationship to circulating monocyte MHC Class II expression, cardiac function and outcome measures.
4. To determine if CPB induces a state of *immune paresis* in the post-operative period.
5. To examine the frequency of polymorphisms amongst candidate genes of inflammation in the bypass population and their effect on circulating cytokine levels and recovery.
6. To identify the mechanism by which MHC Class II expression on monocytes is reduced following cardiopulmonary bypass.

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Materials and General methods

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2.1 INTRODUCTION:

The following chapter describes established scientific methods that have been used during this PhD. Some of these methods have been optimized for use in children, in particular modifying techniques to obtain reproducible results when only 1-2 ml of blood was available. The list of reagents including antibodies, solutions and buffers are listed in this section.

2.2 MATERIALS AND REAGENTS:

2.2.1 Chemicals

Chemical	Supplier
Agarose	Invitrogen/Life Technologies, Paisley, UK
Bovine serum albumin (fraction V)	Sigma, Poole, UK
Cell lysis	BD Bioscience, Oxford, UK
Cell fix (1% formaldehyde, 0.1% sodium azide)	BD Bioscience, Oxford, UK
Citi-fluor (Glycerol/PBS Solution)	Citifluor Ltd, UK
Citric acid ($C_6H_8O_7 \cdot H_2O$)	Sigma, Poole, UK
di-Sodium hydrogen phosphate (Na_2HPO_4)	BDH Merck, Poole, UK
DNA ladder	Gibco, Invitrogen/Life Technologies, Paisley, UK
DNTPs (Deoxynucleotide Triphosphates)	Promega, Southampton, UK
DTT	Invitrogen/Life Technologies, Paisley, UK
EDTA (Ethenediaminetetraacetic acid, disodium salt)	Sigma, Poole, UK
Ethanol	Sigma, Poole, UK
Ficoll hypaque gradient (Lymphoprep)	Nycomed (Amersham), Little Chalfont, UK
Fluorescein isothiocyanate (FITC)	Sigma, Poole, UK
Foetal Calf Serum (FCS)	Sigma, Poole, UK
Gel loading solution	Sigma, Poole, UK
Glycerol	Sigma, Poole, UK

Chemical	Supplier
GM-CSF (Granulocyte-Monocyte Colony stimulating factor)	Schering-Plough, Kenilworth, NJ, USA
Heparin 5000U/ml (preservative free)	CP Pharmaceuticals, Wrexham, UK
Hydrogen Peroxide	Sigma, Poole, UK
Interleukin-10 (IL-10)	NIBSC, Hertfordshire, UK
Interferon gamma (IFN- γ)	NIBSC, Hertfordshire, UK
Magnesium Chloride (MgCl ₂)	BDH Merck, Poole, UK
Monocyte mRNA isolation kit	Dynal Biotech, Bromborough, UK
Oligonucleotide sequences (customised)	Applied Biosystems, Cheshire, UK
Paraformaldehyde (CH ₂ O) _n	Sigma, Poole, UK
Permeabilisation Liquid	Caltag, Towcester, UK
Percoll	Amersham, Little Chalfont, UK
Phosphate buffered saline (PBS) tablets	Oxoid, Basingstoke, UK
Potassium di-hydrogen phosphate (KH ₂ PO ₄)	BDH Merck, Poole, UK
Potassium Chloride (KCl)	BDH Merck, Poole, UK
Primers for TaqMan	Applied Biosystems, Cheshire, UK
Probes (fluorescent) for TaqMan	Applied Biosystems, Cheshire, UK
RPMI 1640 medium	Invitrogen/Life Technologies, Paisley, UK
Saponin	Sigma, Poole, UK
Sodium Azide (NaN ₃)	BDH Merck, Poole, UK
Sodium Chloride (NaCl)	BDH Merck, Poole, UK
Streptavidin-HRP	BD Biosource, Oxford, UK
Sulphuric Acid	BDH Merck, Poole, UK
Superscript Reverse transcriptase	Invitrogen/Life Technologies, Paisley, UK
Taq Polymerase	Promega, Southampton, UK
TEMED	Sigma, Poole, UK
TMB tablets	Sigma, Poole, UK
To-pro-3 iodine (642/661)	Molecular probes, Ludwig Institute, London
Tris-HCl	BDH Merck, Poole, UK
Trypan Blue	Invitrogen/Life Technologies, Paisley, UK
Tumour necrosis factor alpha(TNF- α)	NIBSC, Hertfordshire, UK
Tween 20 (Polyoxethylene sorbitan monolaurate)	Sigma, Poole, UK

2.2.2 Buffers

All solutions were made up to 1000ml in Milli-Q water unless otherwise indicated.

Buffer or Solution	Composition	Concentration	pH
Blocking Solution for ELISA	Coating Buffer (as below) Bovine Serum Albumin	0.5% (5g/litre)	7.4
Citrate Buffer for ELISA	Citric Acid	0.1 M (21g/litre)	5.0
Coating Buffer for ELISA (Phosphate buffered saline)	Sodium Chloride Potassium Chloride di-Sodium hydrogen phosphate Potassium di-hydrogen phosphate	140 mM (8g/litre) 2.7 mM (0.2g/litre) 8.0 mM (1.42g/litre) 1.5mM (0.2g/litre)	7.4
Diluent for ELISA	Blocking Solution (as above) Tween 20	0.01% (v/v)	7.4
FACS washing buffer	PBS (1x) Sodium azide Bovine Serum Albumin	0.02% 0.5% (5g/litre)	
Lysis/Binding buffer for RNA isolation	Tris-HCL LiCL EDTA LiDS Dithiothreitol (DTT)	100 mM 500 mM 10 mM 1% 5 mM	7.5 8.0
MACS buffer	PBS (1X) EDTA Bovine Serum Albumin	2 mM 0.5%	
Formamide (MADGE) Dye	Deionised formamide xylene cyanol bromophenol blue EDTA 0.5M	49mls 12.5mg 12.5mg 1ml	7.9
Phosphate Buffer for ELISA	di-Sodium hydrogen phosphate	0.2 M (28.5g/litre)	5.2

Buffer or Solution	Composition	Concentration	pH
Phosphate Buffered saline (PBS)	PBS tablet	1 per 100 MilliQ	
Phosphate Citrate buffer	Citrate Phosphate r	0.05M 0.025M	5
PBS/BSA buffer for RNA isolation	Sodium Chloride di-Sodium hydrogen phosphate Potassium di-hydrogen phosphate Bovine serum albumin Sodium Citrate	140 mM (8.1g/litre) 8.0 mM (0.98g/litre) 1.5mM (0.16g/litre) 0.1% (w/v) 0.6% (w/v)	7.4
Polmix	Potassium Chloride Tris HCl Gelatin dATP,dGTP,dTTP,dCTP	50mM 10mM 0.001% 0.2mM of each	8.3
Polyacrylamide Gel	10x TBE 30% acrylamide-bisacrylamide (ratio 19:1) N'-tetramethylethylenediamine 25% ammonium per sulphate	5ml 12.5ml 150µl 150µl	
Sticky Silane	ETOH Glacial Acetic Acid γ-methacryloxypropyl-trimethoxy silane EDTA 0.5M	99% 0.05% 0.05% 0.01%	7.9
Stop Solution	di-Hydrogen Sulphate	4 N	
TMB ELISA substrate buffer	Phosphate/Citrate buffer TMB 30% hydrogen peroxide	10ml 1mg 2µl	

Buffer or Solution	Composition	Concentration	pH
Tris-Boric acid-Ethylenediaminetetraacetic acid (10x TBE)	Trizma Base	0.9M	
	Boric Acid	0.9M	
	EDTA	0.2M	
Wash Buffer for ELISA	Sodium Chloride	0.15M	7.4
	Tween 20	0.01% (v/v)	
Wash Buffer A for RNA isolation	Tris-HCl	10 mM (pH 7.5)	
	LiCl	0.15 M	
	EDTA	1 mM	
	LiDS	0.1%	
Wash Buffer B for RNA isolation	Tris-HCl	10 mM (pH 7.5)	
	LiCl	0.15 M	
	EDTA	1 mM	

2.2.3. Antibodies

All antibodies used for flow cytometry or confocal microscopy are listed here. These include mouse monoclonal anti-human antibodies, isotype control antibodies, and one secondary antibody raised in goat. Where available, the table includes the species in which the antibodies were derived, and the clone.

Antibody	Fluorescence	Isotype	Clone	Supplier	Code
CD14	-	mouse IgG2a	TÜK4	Dako	M0825
CD14	RPE	mouse IgG1	TÜK4	Dako	R0864
CD14	RPE-Cy5	mouse IgG2a	TÜK4	Serotec	MCA1568C
CD64	RPE –Cy5	mouse IgG1	10.1	Dako	C7220
HLA-DP	-	mouse IgG1	HI43	Pharmingen	32431 A
HLA-DQ	-	mouse IgG2a	TÜK169	Pharmingen	32421A
HLA-DR	-	mouse IgG2a	G46-6	Pharmingen	34231A
HLA-DR	FITC	mouse IgG1	G46-6	Pharmingen	555811
HLA-DP, -DQ, -DR	-	mouse IgG1	CR3/43	Dako	F0816
HLA-DP, -DQ, -DR	FITC	mouse IgG1	CR3/43	Dako	F0817

IgG1 isotype control	FITC	mouse	-	Dako	X0927
IgG1 isotype control	RPE	mouse	-	Dako	X0928
IgG2a isotype control	FITC	mouse	-	Dako	X0933
IgG2a isotype control	RPE	mouse	-	Dako	X0950
Goat-Anti-mouse IgG	Texas Red-X	-	-	Molecular Probes	T-6390

2.2.4 Antibodies and Standards for ELISAs

Antibody pairs and standards utilised for ELISA's are listed below.

Antibody	Isotype	Clone	Supplier	Code
Human TNF-α CytosetsTM			Biosource	CHC1754
Capture TNF- α	mouse IgG1 & IgG2	68B6A3 & 68B2B3	Biosource	58.175.08
Detection TNF- α	mouse IgG1	68B3C5	Biosource	58.175.02
Recombinant human TNF- α			Biosource	58.175.10
Human IL-10 CytosetsTM			Biosource	CHC1324
Capture IL-10	mouse IgG1	945A5D11	Biosource	58.132.08
Detection IL-10	mouse IgG1	945A5A10	Biosource	58.132.02
Recombinant human IL-10			Biosource	58.132.10
Human IL-8 CytosetsTM			Biosource	CHC1304
Capture IL-8	mouse IgG1	893A6G8	Biosource	58.130.08
Detection IL-8	mouse IgG1	790A28G2	Biosource	58.130.02
Recombinant human IL-8			Biosource	58.130.10
Human IL-6 CytosetsTM			Biosource	CHC1264
Capture IL-6	mouse IgG1	677B6A2	Biosource	58.126.08
Detection IL-6	mouse IgG1	505E23C7	Biosource	58.126.02
Recombinant human IL-6			Biosource	58.126.10
Human IL-1ra CytosetsTM			Biosource	CHC1184
Capture IL-1ra	mouse IgG1	998A2A2	Biosource	58.118.08
Detection IL-1ra	mouse IgG1	A71B6D11	Biosource	58.118.02
Recombinant human IL-1ra			Biosource	58.118.10

2.2.5 General Equipment and Consumables

Equipment	Supplier
Beckman's 96 well array plates	
Bijou (5ml)	SLS, Wilford, Nottingham, UK
Biohit repeating dispenser	Alpha Laboratories, UK
Confocal Microscope	Leica Microsystems, Milton Keynes, UK
Coverslips for microscopy	BDH Merck, Poole, UK
Dako Pen for Immunohistochemistry	Dako, Cambridge, UK
Dynal Magnet	Dynal Biotech, Bromborough, UK
Dynal magnetic particle concentrator	Dynal Biotech, Bromborough, UK
Dynatech MRX ELISA plate reader	Dynex technologies, UK
FACScalibur Flow Cytometer	Becton Dickinson (BD Bioscience) Oxford, UK
FACScan Research and Lysis II Software	Becton Dickinson (BD Bioscience) Oxford, UK
FACs tubes	Becton Dickinson (BD Bioscience) Oxford, UK
Falcon tubes (5ml)	BD Falcon, BD Bioscience, Oxford, UK
Falcon tubes (15ml)	TPP, Trasadingen, Switzerland
Falcon tubes (50ml)	TPP, Trasadingen, Switzerland
Finnipipette multichannel dispenser	Life Sciences International
Macs CD14 ⁺ magnetic MicroBeads	Milteny Biotech, Bisley, UK
Macs Column	Milteny Biotech, Bisley, UK
Macs Mini Magnet	Milteny Biotech, Bisley, UK
Macs Magnet Stand	Milteny Biotech, Bisley, UK
MADGE Perspex forma	MadgeBio Ltd, Huntingdon, Cambridgeshire, UK
Maxisorb ELISA plates (96 well)	Nalgene NUNC, Rochester, NY, USA
Mikrotek ELISA Software	Dynatech Laboratories, Chantilly, VA, USA
MJ Tetrad DNA Engine Thermocycler	MJ Research, UK

Equipment	Supplier
MRX ELISA microplate reader	Dynatech Laboratories, Chantilly, VA, USA
96-well microtiter plate (Omniplate)	Hybaid, Thermolife Sciences, UK
Oligo (dT)25 magnetic Dynabeads	Dynal Biotech, Bromborough, UK
96-well polycarbonate PCR plates (Model M)	Corning Costar, High Wycombe, UK
Slides- poly-L-lysine coated for Microscopy	BDH Merck, Poole, UK
Syringe filters 0.22µm	Millipore, Watford, UK
TaqMan sequence detection system (ABI Prism 7000 SDS Software)	Applied Biosystems, Cheshire, UK
Tecan GENios 96 well absorbance/fluorescence reader	Tecan, Reading, UK
Universal tubes (20ml)	Helena Bioscience, UK
UVP Gel Documentation System	Synoptics Ltd, Cambridge, UK

2.3 PATIENT DATA COLLECTION:

In each of the following chapters, results are presented from infants and children undergoing cardiopulmonary bypass. In order to interpret results, detailed and accurate phenotypic data on each patient, the operation undertaken, and their post-operative recovery was required (Appendix I). Up until the time of this project, there was no one source within the hospital that recorded this data collectively. The patient data presented in the following chapters was the result of clinical review of the patient, review of the anaesthetic and operative records, the surgical database maintained by cardiothoracic surgery, and daily review of the Intensive Care bedside electronic charting system (Careview, Hewlett Packard, USA).

Combining the patient details with the scientific data resulted in an extremely large database that was beyond the capacity of a standard spread sheet. Statistical analysis also required cross referencing data from various areas of data collection. In order to achieve these requirements, a purpose designed Access Data Base (Microsoft Office'98/XP) was developed over the first 6 months of this PhD. Results from each study were then entered into the databases and queries run to address each of the original study hypotheses. Results from the queries were imported into Statistical Package for the Social Sciences (SPSS 8.0) for statistical analysis. Graphs were drawn using SPSS, Excel (Microsoft Office'98/XP), or PRISM 3.0 (GraphPad Software Inc).

Each study undertaken was approved by the local Research Ethics Committee and informed written consent was obtained from the parents of all participating children. Patient data was password protected and managed according to the data protection policy of University College London.

2.4 BLOOD COLLECTION AND HANDLING

Whole blood was collected from subjects via an arterial line after discarding the initial 3mls as heparin rich dead space. Samples were placed immediately into sterile 5ml bijoux containing preservative free heparin (final concentration 10 U/ml). To avoid *ex*

in vivo activation, all blood samples were processed immediately. When blood was required for genetic analysis, a second sample anticoagulated with 4.2 mM ethylenediaminetetraacetic acid (EDTA) was collected and samples stored at -70°C until required.

2.4.1 Separating monocytes from whole blood

For several experiments a more concentrated form of monocytes was required than that provided by whole blood. In order to achieve this, density gradients and positive selection methods were utilised.

2.4.1.1 Isolation of peripheral blood mononuclear cells (PBMC's)

Lymphocytes and monocytes (PBMC's) were isolated from whole blood by centrifugation over a density gradient (Fig 2.1). Anticoagulated whole blood was diluted with an equal volume of RPMI 1640 medium, layered over a Ficoll hypaque gradient (density 1.077g/ml) and centrifuged at 400g for 25 minutes at room temperature. The cells present at the interface were recovered and washed twice with RPMI 1640 medium supplemented with 5% foetal calf serum (FCS). Cell numbers were counted using a haemocytometer (Weber, UK) and concentration (cells/ml) recorded.

2.4.1.2 Isolation of monocytes

Where a pure population of monocytes was required, PBMC's were further purified by either a secondary density gradient or positive selection on a MACS column (Figure 2.1). The two methods were assessed for purity when small volumes of whole blood were utilised.

2.4.1.2.1 *Standard Isotonic Percoll Gradient (SIP)*

Standard Isotone Percoll was prepared by adding 1 part phosphate buffered saline (PBS) (10x) to 9 parts Percoll (density 1.793 g/ml). The SIP was then diluted to 3 concentrations using RPMI 1640 with 5% FCS:

60% SIP

47.5% SIP

34% SIP

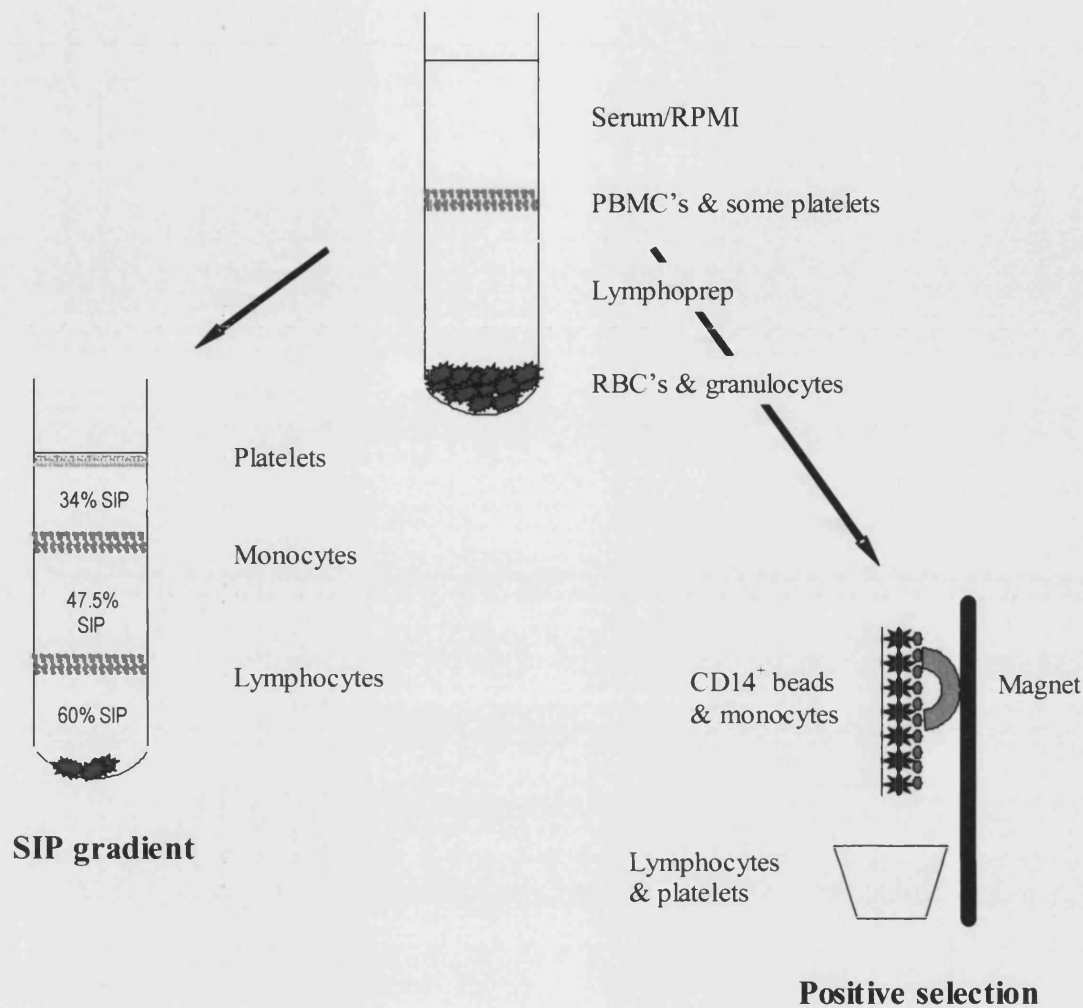


Figure 2.1: Isolation of Monocytes from whole blood.

Monocytes and lymphocytes (PBMC's) were recovered from whole blood at the serum-lymphoprep interface after centrifugation over a Ficoll hypaque density gradient. PBMC's were further purified by either a second density gradient or positive selection using CD14⁺ labelled magnetic beads (~ 50nm in size).

PBMC's were resuspended in 2ml of 60% SIP. Then 4.5ml of 47.5% SIP was layered carefully over the 60%, followed by 2ml of the 34% SIP. The tube was centrifuged at 1750g for 45 minutes at room temperature, before the interface between the 34% and 47.5% SIP was recovered. Monocytes recovered from this interface were washed a further 3 times using cold RPMI with 5% FCS, counted and concentration recorded.

The purity & activation status of the monocyte preparation was checked using flow cytometry. When ≥ 20 ml of whole blood was used, purity was typically $>95\%$.

2.4.1.2.2 *Positive selection using CD14 beads*

PBMC's isolated from 2-3ml of whole blood were re-suspended in 25 μ l MACS buffer and incubated on ice for 15 minutes with 6 μ l of magnetic MicroBeads labelled with a CD14⁺ monoclonal antibody. The solution was then passed through a matrix filled column lying within a magnetic field. Cells binding to the magnetic labelled CD14⁺ antibody were retained and the remaining cells washed away with MACS buffer (x2). The column was removed from the magnetic field and washed a 3rd time with MACS buffer to release the CD14⁺ cells. A cell count was performed and cells were assessed by flow cytometry to note any change in surface expression of MHC Class II, and confirm a purity of $\geq 95\%$.

2.5 FLOW CYTOMETRY

Flow cytometry allowed identification of monocytes from whole blood samples based on the cell size and granularity, in addition to the presence of fluorescence labelled monoclonal antibody to CD14. Flow cytometry was used throughout this work to quantitate monocyte expression of MHC Class II. Whilst specific details are given in the relevant chapters, the general principals of antibody staining and data collection using flow cytometry are outlined here.

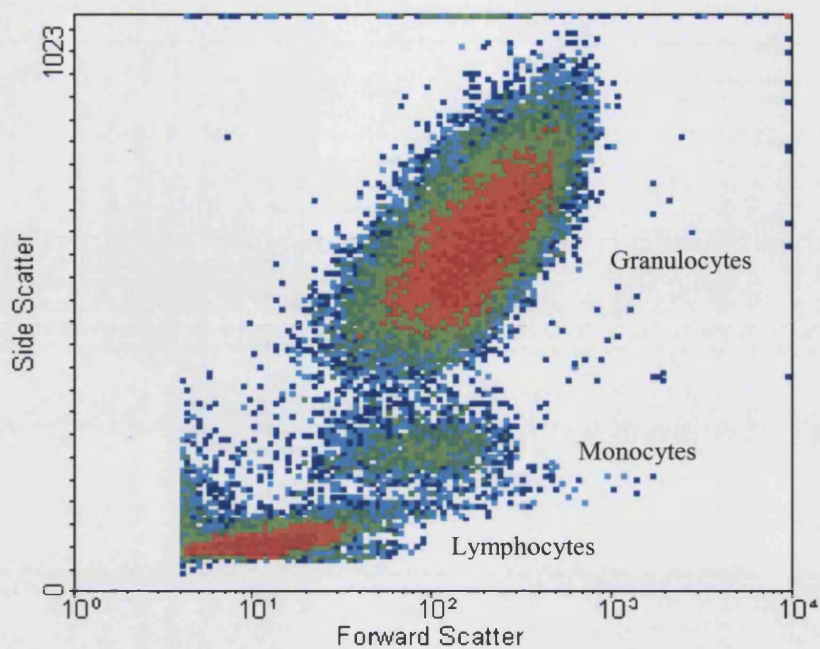


Figure 2.2 Flow cytometric profile of whole blood after lysis of red blood cells.

Anticoagulated whole blood was incubated with the appropriate monoclonal antibodies before the red blood cells are lysed and the remaining cells fixed. The sample was then passed through the flow cytometer where each particle was assessed and plotted according to its size (forward scatter) and granularity (side scatter). Monocytes were readily recognisable as the smaller cloud of cells located between granulocytes and lymphocytes.

2.5.1 Surface Staining

Whole blood was incubated with the appropriate fluorescent monoclonal antibody/antibodies at room temperature, in the dark for 10-15 minutes. A final antibody concentration of $5\mu\text{g/ml}$ or $0.5\mu\text{g}/10^6$ cells was used unless otherwise specified. For each antibody an isotype matched control was used to control for non-specific binding. One ml of cell lysis buffer was added to lyse red blood cells, before the remaining cells were pelleted by centrifuging at 350g for 5 minutes. The supernatant was discarded and the pellet resuspended in 200-300 μl of cell fix solution. To conserve fluorescence, samples were protected from the light and stored at 4°C until analysed.

[illegible]

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme-linked immunosorbent assays (ELISA) were used to measure cytokines in plasma and culture supernatant. The protocol was based on manufactures instructions but optimised further to maximise detection of cytokines at low concentrations.

High-binding 96-well plates were coated with 100µl/well of the capture antibody of choice diluted to 1µg/ml (except TNF-α which was diluted to 5µg/ml) with Coating Buffer A, covered and left at 4°C for 12–18 hours. Coating antibody was aspirated from the wells and each well washed once with wash buffer before 300µl/well of blocking solution was added. Plates were incubated for a minimum of 2 hours at room temperature (18-25°C). The block was aspirated and wells washed 4x before plasma samples and serially diluted standards were added at 100µl/well (Table 2.1).

Cytokine Standard	Range
TNF-α standard curve	10,000pg/ml to 5pg/ml
IL-10 standard curve (stimulated)	5,000pg/ml to 1pg/ml
IL-8 standard curve (stimulated)	10,000pg/ml to 1pg/ml
IL-6 standard curve (stimulated)	10,000pg/ml to 2.5pg/ml
IL-1ra standard curve (stimulated)	10,000pg/ml to 2.5pg/ml

Table 2.1 Ranges of Cytokine Standard Curves. Standard curves for the cytokines of interest were created using 12 serial dilutions over a range of concentrations.

Attempts to optimise a standard curve starting with a top standard of < 5,000pg/ml resulted in loss of the sigmoid curve with no improvement in discrimination at lower concentrations. Samples were added neat except IL-1ra, which was diluted 1 in 10. Where stimulated samples were off the upper limits of the standard curve all samples for that patient and cytokine were re-run at a dilution of 1 in 10. A substrate blank was used on all plates, and all standards, samples and blanks were assayed in duplicate.

Plates were covered and left overnight at 4°C. Wells were aspirated and washed 4 times, following which the respective biotinylated antibody diluted (0.1-0.4µg/ml) in diluent was added (50µl/well). Plates were incubated at room temperature on a rotational shaker for 2 hours. After 4 washes, 100µl/well of Streptavidin-horse radish peroxidase (HRP) 1/2500 was added and plates incubated for a further 30-45 minutes at room temperature with continual shaking. Following 4 washes, tetramethylbenzidine (TMB) substrate was added to each well (100µl/well). Plates were protected from light and incubated at room temperature with continued shaking. The colour reaction was stopped after 45-60 minutes (time individualised to each plate) with 4N Sulphuric Acid (100µl/well) and optical densities of each well were measured using a Dynatech MRX ELISA plate reader at 450nm (reference filter 650nm). Cytokine concentrations in the unknown sera were read from the curve generated from a known standard included on each plate (Fig 2.3). This technique allowed confidence to a lower detection range of between 5-10pg/ml for TNF-α and 3-5pg/ml for IL-10, -8, -6, and -1ra respectively.

To determine assay precision, the coefficient of variation (standard deviation of the replicate responses x 100 / mean of the response) was determined using replicas on one plate (intra-plate precision) as well as multiple plates (inter-plate precision). The highest coefficient of variation for any of the assays used was 4.2 for intra-plate precision (n=4), and 8.8 for inter-plate precision (n=4).

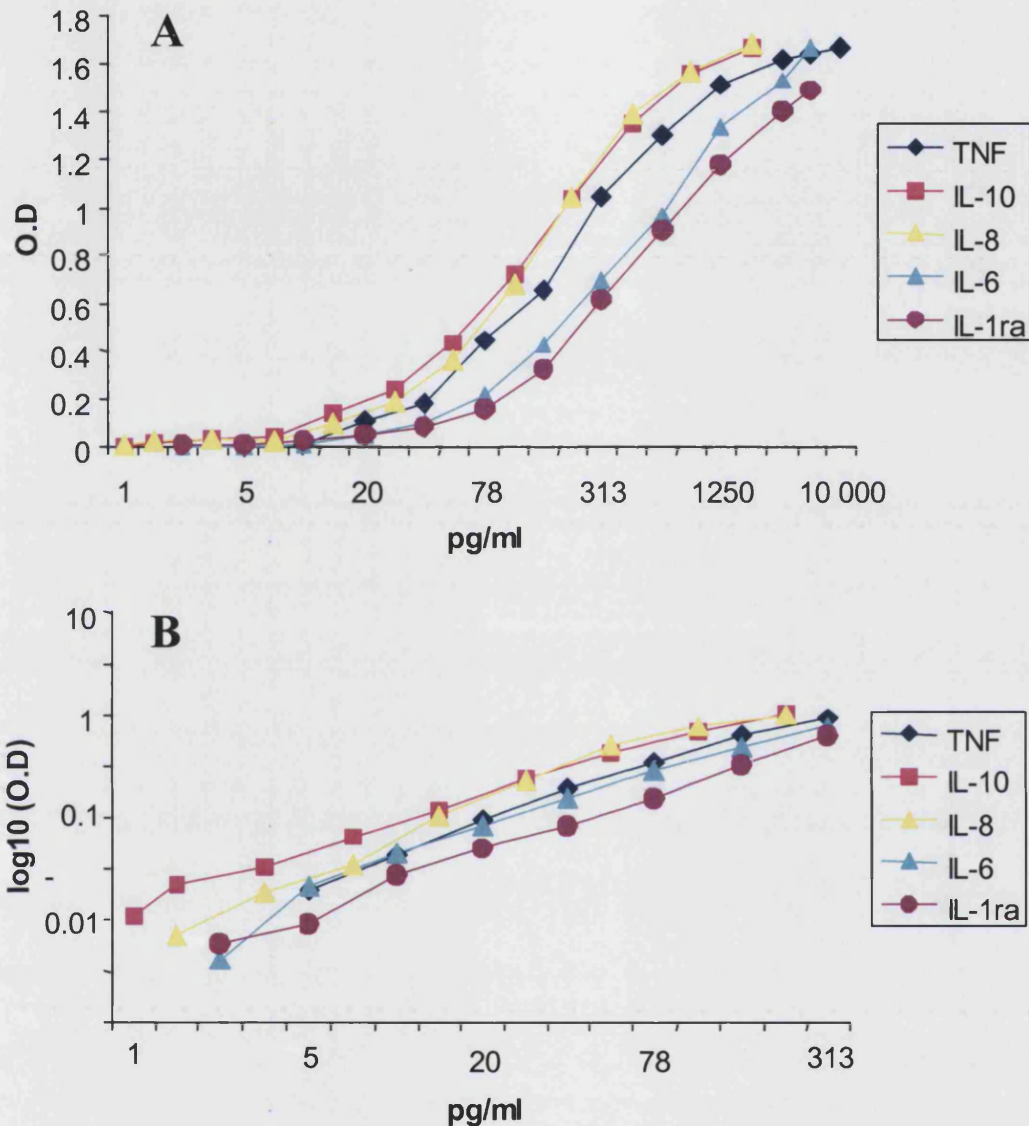


Figure 2.3a & b: Standard Curves for Cytokine ELISA's

Standard curves for each cytokine were generated by 1:2 serial dilutions of a known concentration of human recombinant protein. The optical densities were measured on a Dynatech MRX plate reader and analysed by sigmoid (2.6a) and quadratic regression (2.6b) to generate 2 standard curves. All serum cytokine concentrations for a single patient were calculated from one of the two curves depending on the range of concentrations. Results were read from the standard curve generated by quadratic regression if all values in a patient's time course were $< 300\text{pg/ml}$.

2.6.1 General scheme for human cytokine ELISA

CAPTURE ANTIBODY	Mouse monoclonal anti-Human IgG1 antibody on Maxisorp plates (12-18hrs 4°C)
	↓
<i>Wash</i>	Saline with Tween 20 (x1)
	↓
BLOCK	0.5% BSA in PBS (2hrs RT)
	↓
<i>Wash</i>	Saline with Tween 20 (x4)
	↓
SERA	Sera (neat or diluted 1/10 in 0.5% BSA-PBS-T) (overnight 4°C)♣
	↓
<i>Wash</i>	Saline with Tween 20 (x4)
	↓
DETECTION ANTIBODY	Biotinylated mouse monoclonal anti-Human IgG1 antibody (2hrs RT)♣
	↓
<i>Wash</i>	Saline with Tween 20 (x4)
	↓
STREPTAVIDIN	Streptavidin-HRP in PBS (30-45min RT)
	↓
<i>Wash</i>	Saline with Tween 20 (x4)
	↓
SUBSTRATE	TMB in PO ₄ /citrate/H ₂ O ₂ (45-60 min RT covered)♣
	↓
STOP	2N H ₂ SO ₄
	↓
COLOUR	Absorbance 450nm

♣ denotes steps differing from manufacturers recommendations discussed in detail in section 4.4.1

2.7 GENOTYPING FOR CYTOKINE PROMOTER POLYMORPHISMS

2.7.1 DNA extraction and standardisation

A single sample of 1-2mls of whole blood was collected in 4.2mM EDTA for each patient, and DNA extracted in batches by the genetics laboratory at Great Ormond Street Hospital using established techniques. Prior to standardisation, 3 random samples of DNA were selected from each batch and UV quantification performed using a 1:1, 1:9, 1:19 and 1:99 dilution to determine the dilution which fell within the linear part of the standard curve. DNA samples were then appropriately diluted (1:19) with double deionised water (dd-H₂O) on 96-well plates, and optical densities read using a spectrophotometer (Tecan Genios 96 well absorbance/fluorescence reader, TECAN, Austria,). The concentration of DNA in each sample was calculated using the formula below, and 96 x 150µl *stock arrays* of DNA standardised to a concentration of 15ng/µl were created.

$$[\text{DNA}] \text{ present} = \text{Optical density (260 wavelength)} \times \text{dilution (1:19)} \times 50$$

Each array was clearly labelled and had a corresponding grid sheet listing samples by study number within an 8x12 grid (Fig 2.4). All arrays had a minimum of 3 blank wells. From the stock arrays, 2 x 96-well *working arrays* were made each with 25µl/well. The creation of working arrays, minimised risk of contamination of stock DNA, and reduced the number of freeze/thaw episodes for the total DNA. All arrays were stored at -80°C. Creation of 96 well arrays facilitated higher throughput and minimised risk of contamination of stock DNA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	PC
F	6	14	22	30	38	46	54	62	70	78	86	Blank
G	7	15	23	31	39	47	55	63	71	79	87	Blank
H	8	16	24	32	40	48	56	64	72	80	88	Blank

Figure 2.4: An example of a standard array grid sheet. Each plate contained at least 3 blanks, and space for a positive control (PC).

2.7.2 Polymerase Chain Reaction (PCR)

The technique of PCR relies on the fact that a DNA strand can be denatured into single strands by heat, and will anneal with primers and nucleotide bases to reform a double strand on cooling. Primers complimentary to sections of DNA on either side of the sequence to be studied bind to the genomic DNA, and a DNA polymerase adds nucleotides base by base between the 2 primers. The polymerase used in PCR is derived from the bacterium *Thermus aquaticus* (*Taq*) and is heat stable, thus replenishment of polymerase after each cycle of heating and cooling was not required. PCR enabled very small initial amounts of DNA to be increased in quantity until there was sufficient DNA for genotyping to be performed.

The primer sequences and PCR cycle conditions specific to each PCR are detailed in Tables 2.1 and 2.3.

Polymorphism	Oligonucleotide Sequence 5'-3' (Forward / Reverse)	Fragment Size (bp)
IL-6 -174 G/C	5'-TGA CTT CAG CTT TAC TCT TGT-3' 5'-CTG ATT GGA AAC CTT ATT AAG-3'	190
IL-10 -1082 G/A	5'-CCA AGA CAA CAC TAC TAA GGC TCC TTT -3' 5'-GCT TCT TAT ATG CTA GTC AGG TA-3'	377
IL-10 -819 C/T	5'-CAA CTT CTT CCA CCC CAT CTT T-3' 5'-GTG GGC TAA ATA TCC TCA AAG TT-3'	477
IL-10 -592 C/A	5'-CAA CTT CTT CCA CCC CAT CTT T-3' 5'-GTG GGC TAA ATA TCC TCA AAG TT-3'	477
TNF- α -308 G/A	5'-GGA GGC AAT AGG TTT TGA GGG cCA T-3' 5'-CCT TGG TGG AGA AAC CCA TGg GCT-3'	253

Table 2.2: Primer pairs for allele-specific restriction enzyme analysis

The sequences of primers used for each of the cytokine promoter polymorphisms studied and size of fragment generated is shown. Sequences are in 5'-3' direction. Sequences of the upstream primer are shown above the sequence of the downstream primer. Primer nucleotides that differ from the genomic sequences are in lower case letters.

2.7.2.1 Sample preparation for PCR

Working arrays were defrosted and centrifuged at 1450g for 1 minute. This minimised DNA loss or cross-well contamination on removal of array lid. Two microlitres of each DNA sample was removed and transferred into a 96-well polycarbonated PCR plate using a multichannel Finnipipette. Positive and negative controls were used in each array to ensure accuracy. Extreme care was taken to ensure that samples were placed in the identical orientation as in the original array. Loaded PCR plates were then centrifuged at 160g for 1 minute to ensure the DNA was at the bottom of each well, before drying on a Thermal Cycler block at 80°C for 10 minutes.

Polymerase chain reactions were performed in a total volume of 20 μ l. Each reaction was prepared on ice and contained 1x concentration of polmix (50mM KCl, 10mM Tris-HCl (pH 8.3), 0.2mM dATP, dGTP, dTTP and dCTP), 1.5-2.5mM MgCl₂, 600-1000pmol of forward and reverse primers, and 25-30 units of *Taq* polymerase. The magnesium concentration was optimised for each PCR (see Chapter 6, section 6.4.2).

The PCR mix was added to each well of the PCR plate using an automatic Biohit repeating dispenser. Each sample was overlaid with 20 μ l of paraffin oil to prevent evaporation. PCR amplification was performed on an MJ Tetrad DNA engine Thermocycler, using cycle conditions specific to each PCR. Presence of product was confirmed on a 2% agarose gel stained with ethidium bromide.

Polymorphism	Step 1 (denature)	Step 2	Step 3	Step 4	No. of cycles (steps 2-4)	Termination step
IL-6 -174 G/C	95°C 4 min	95°C 40 sec	50°C 30 sec	72°C 1.5 min	35 cycles	72°C 5 min
IL-10 -1082 G/A	95°C 5 min	95°C 45 sec	59.8°C 45 sec	72°C 45 sec	30 cycles	72°C 10 min
IL-10 -819 C/T	95°C 5 min	95°C 45 sec	65°C 45 sec	72°C 45 sec	30 cycles	72°C 10 min
IL-10 -592 C/A	95°C 5 min	95°C 45 sec	65°C 45 sec	72°C 45 sec	30 cycles	72°C 10 min
TNF-α -308 G/A	95°C 5 min	95°C 45 sec	57°C 45 sec	72°C 45 sec	30 cycles	72°C 10 min

Table 2.3: PCR cycle conditions for each promoter polymorphism

2.7.3 Restriction Enzyme Digest

Restriction enzymes are enzymes derived from bacteria that will cleave double stranded DNA at a particular sequence. The restriction enzyme is sensitive even to a single base change in the recognition sequence, and thus can be used to detect point mutations and single base polymorphisms. A single base change, such as those seen in the promoter polymorphisms studied, eliminate or create a cutting site for the restriction enzyme.

Following generation of the PCR product, a restriction enzyme digest mix was made containing sufficient enzyme to digest the PCR product. 8-10 μ l of PCR mix was added to 5 μ l of digest mix in a 96-well microtiter plate, and the plate centrifuged at 210g for 1 minute. The PCR/digest mix was incubated for a minimum of 4 hours at a temperature appropriate for the specific enzyme used (Table 2.4).

2.7.4 Microtitre Array Diagonal Gel Electrophoresis (MADGE)

The DNA fragments produced by restriction enzyme digest were separated using electrophoresis on a non-denaturing polyacrylamide gel, using a technique known as microtitre array diagonal gel electrophoresis (MADGE). This technique made it possible to electrophorese the 96 wells of a standard PCR plate on a single gel by running the samples diagonally. This allowed the 96 well DNA array format to be retained throughout the genotype processing.

MADGE gels consist of an open arrangement of 8 x 12 wells each 2mm deep. The wells are arranged at an angle of 71.2° to the short axis of the array, but perpendicular to the long-axis of the Perspex formers used. Thus the maximum track length for genotype resolution was 26.5mm (Fig 2.5).

Polymorphism	Restriction Enzyme (units/sample)	Manufacturer	Units / sample	Temperature of digest	Sequence recognised	Fragments generated (bp)
IL-6 -174 G/C	Nla III	New England Biolabs	0.5	37°C	CATG↓	-174 G – 190 bp -174 C – 143 / 47 bp
IL-10 -1082 G/A	EcoN1	New England Biolabs	3	37°C	CCTNN↓NNNAGG	-1082 G – 253 / 97 / 27 bp -1082 A – 280 / 97 bp
IL-10 -819 C/T	Mae III	Roche Applied Science	0.3	55°C	↓GTNAC	-819 C – 217 / 175 / 85 bp -819 T – 392 / 85 bp
IL-10 -592 C/A	CSP 61	Fermentas	2	37°C	G↓TAC	-592 C – 311 / 116 / 42 / 8 bp -592 A – 240 / 116 / 71 / 42 / 8 bp
TNF-α -308 G/A	NcoI 1	New England Biolabs	4	37°C	C↓CATGG	-308 G – 210 / 23 / 20 bp -308 A – 233 / 20 bp

Table 2.4: Details of Restriction Digests

The details of the specific restriction enzyme and optimal temperature for digest are outlined for each cytokine polymorphism. The sequence recognized by the restriction enzyme, cutting site and size of fragments generated are tabulated in the last 2 columns.

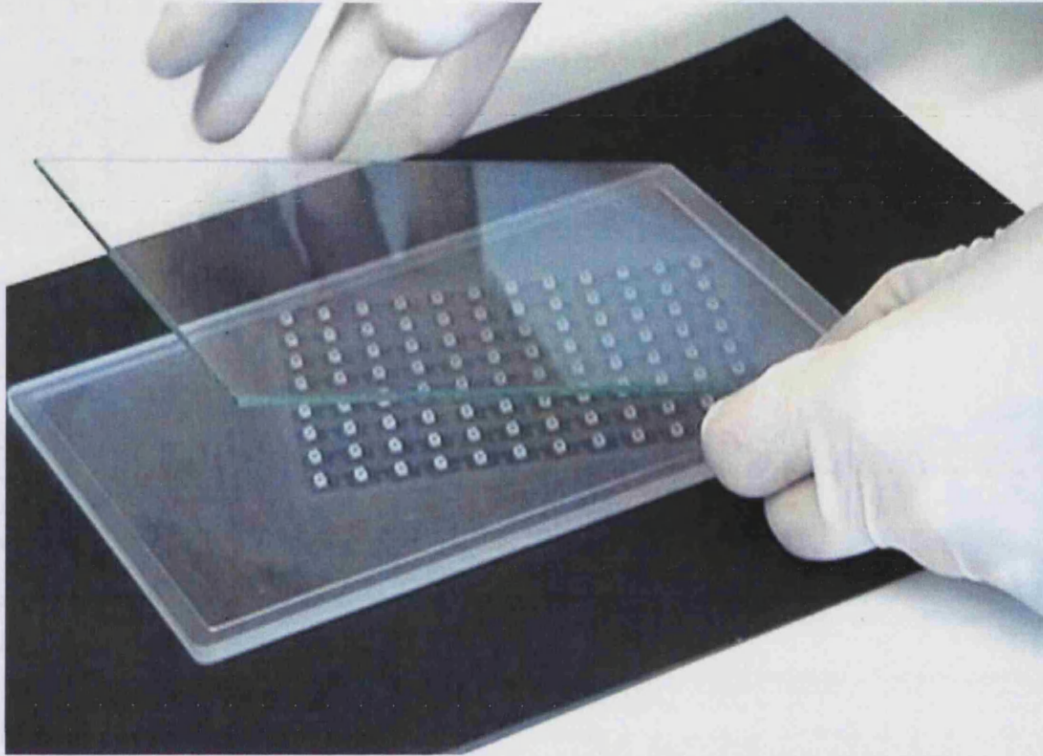


Figure 2.5 Creating a 96 well polyacrylamide gel using the MADGE perspex forma. The polyacrylamide mix is poured onto the forma, and a sticky silane coated glass plate is placed on top. After setting the gel and plate are lifted off the forma and placed in ethidium bromide for staining prior to use.

Six or 7.5% polyacrylamide gels stained with 0.5 μ g/ml ethidium bromide were used for gel electrophoresis. Adherence of polyacrylamide gels to glass plates was enhanced by coating the glass with γ -methacryloxypropyltrimethoxy silane (sticky silane). Five microlitres of digested PCR product was added to 2 μ l of formamide dye prior to loading the polyacrylamide gel. Gels were electrophoresed at 100-120V to separate digest products and visualised using UVP Gel Documentation System (Fig 2.6). Each gel contained a positive and negative control and genotype was checked by 2 individuals. Any conflict in genotype between the 2 observers was resolved by repeated genotyping.

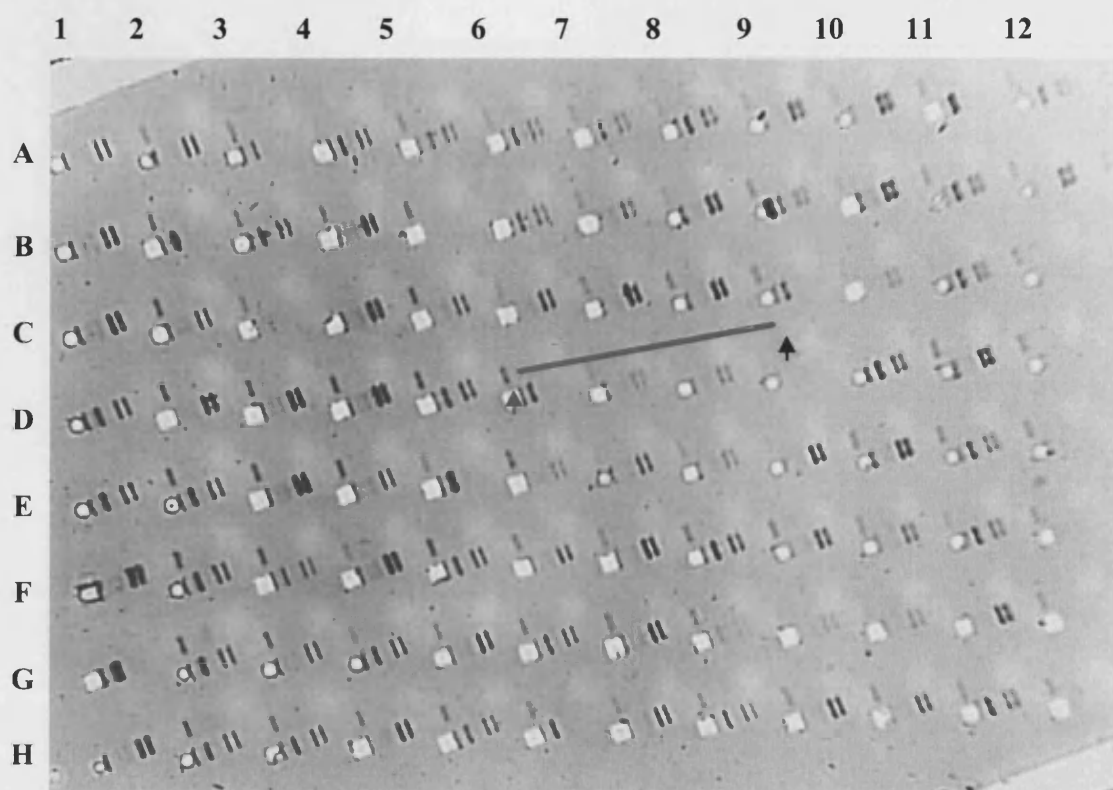


Figure 2.6 Image of a 7.5% polyacrylamide gel with digested PCR product

The microtitre array diagonal gel electrophoresis (MADGE) allowed 96 patient samples to be analysed simultaneously on a horizontal polyacrylamide gel. As each step involves a 96 well template, there is conservation of the patient's position from the stock DNA array through to genotyping. At least 3 blanks were included on every plate (in this gel 12C to 12H), and a positive control (12B). The digested PCR product is loaded into the well marked with a green arrow, and is electrophoresed at an angle along the red line. Very small products (< 50 bp) may travel to the next well (marked with a black arrow) and therefore not be seen.

2.8 CONFOCAL MICROSCOPY

2.8.1 The Microscope

For the final part of this thesis, a microscopic technique was required to complement flow cytometry in establishing the amount and location of MHC Class II. Confocal microscopy was chosen because of its superior ability to differentiate between surface and intracellular fluorescence.

In attempting to localise a molecule to either an intracellular or surface position, it was important not to have surface molecules on either side of the section being superimposed. The combination of a laser and a pinhole avoids the common problem in conventional microscopy of in-focus information being presented simultaneously with defocused information from the same cell, creating a blur. Use of confocal microscopy enabled detailed imaging and semi-quantitation of cellular components that were labelled with fluorochromes.

Confocal images were obtained using a Leica SP2 confocal laser scanning microscope system, and Adobe Photoshop 5.0 software (Adobe, USA) was used to maximise the quality of the digital images where appropriate.

2.8.2 Surface Staining

PBMC's or monocytes were incubated with purified mouse anti-human monoclonal antibody to CD14 ($0.5\mu\text{g}/10^6$ cells) at room temperature for 30-45 minutes. Cells were washed once with FACS wash, and fixed with 2% paraformaldehyde. An appropriate second layer anti-mouse flurochrome was then added (Cy-5) and the sample was incubated in the dark at room temperature for a further 30-45 minutes. Cells were washed and incubated with a primary conjugated antibody to MHC Class II (FITC) or isotype matched control ($5\mu\text{g}/10^6$ cells), for 30-45 minutes.

The sample was washed in FACS wash (x2) and a cell count was performed. The sample was adjusted to 2×10^6 cells per ml, and $50\mu\text{l}$ placed on a polylysine coated microscope slide, air dried and mounted with Citi-fluor. Cells were examined using 100x oil immersion magnification on the confocal microscope.

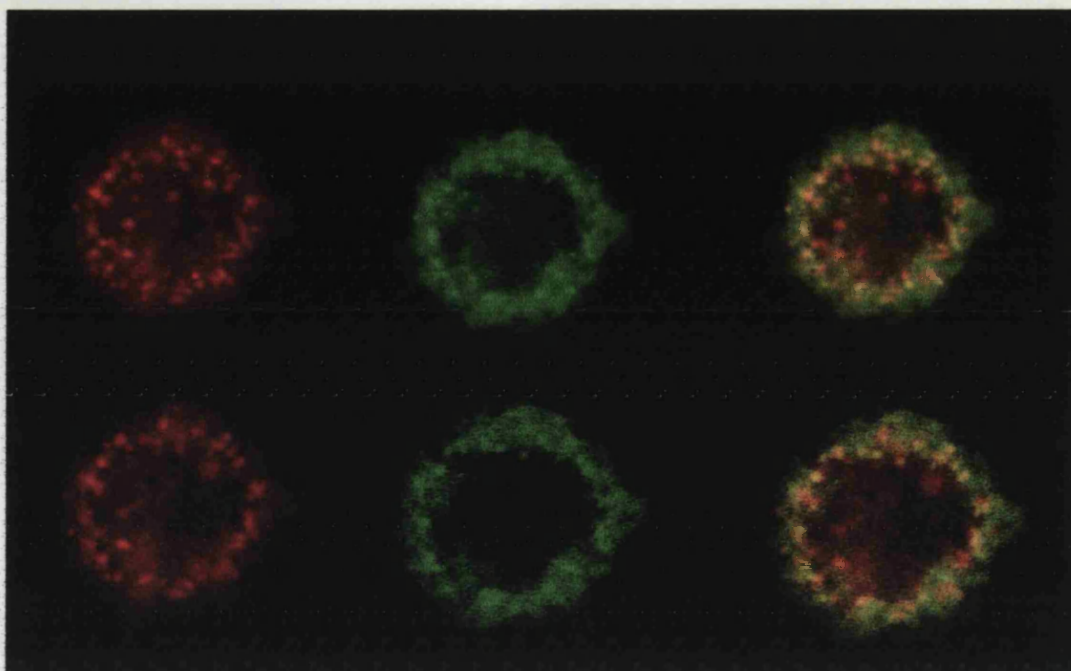


Figure 2.7 Surface staining of monocytes for CD14 and MHC Class II expression. PBMC's isolated by density gradient from 2 patients were incubated with monoclonal antibody to CD14 (2nd layer Cy-5-red) and MHC Class II (FITC-green). Imaging was performed using a confocal immunofluorescence microscope (x100).

2.8.3 Nuclear staining

Where nuclear staining was required, cells were permeabilised after surface staining using either 1% saponin or permeabilisation liquid, and incubated with the nuclear stain To-pro-3 iodine (1:800 dilution). If intracellular staining of the sample was required, To-pro-3 iodine was combined with the flurochrome labelled monoclonal antibody to the intracellular structure of choice. Following 30-45 minute incubation, cells were washed twice and mounted.

2.8.4 Intracellular staining

PBMC's or monocytes were surface stained for CD14 using the two-layer method described above. After fixing, cells were then permeabilised and the fluorochrome labelled antibody to the structure of interest was added. An isotype-matched control was used in a duplicate sample, for each intracellular structure being examined. Following 30-45 minute incubation, cells were washed (x2) and mounted as described above.

2.8.5 Analysis

Slides were stored at -20°C until examined using the confocal microscope. All slides from a single patient were examined at the same time, with the examiner blinded to the flow cytometry and mRNA results. Monocytes were selected based on cell morphology. For each patient, microscope settings were optimised for monocytes from the pre-operative sample and then settings were left unchanged for images acquired from post-operative samples. For each sample 5 monocytes were randomly chosen, examined, and photographed for both surface and intracellular MHC Class II staining. Images were taken at 200x, 400x and 800x magnification.

2.9 STATISTICS

A number of parametric and non-parametric tests were used to analyse the results presented here. A description of individual tests and their application is given in the relevant chapters. Parametric tests were used wherever the data were normally distributed or could be transformed to normality. As much of the data was not normally distributed and group numbers small, non-parametric tests were utilized to detect differences between groups. Results between groups were compared using Mann-Whitney U test, Kruskal-Wallis test, or Spearman's correlation coefficient. The change of the data over the study period was analysed with a one-way analysis of variance for repeated measures (ANOVA) if normally distributed, or the Friedman test if a non parametric equivalent was required.

In an effort to avoid repeated analysis and the associated risk of a chance finding being significant, intergroup comparisons and correlation analyses were only performed on cytokine samples collected at end of surgery, 8, 24, and 48 hours post operatively. These samples were chosen for based on previous reports in the literature (Wernovsky, Wypij and others, 1995). Analysis was performed using the Statistical Package for the Social Sciences (SPSS 10.0). A *p* value less than 0.05 was considered significant

CHAPTER 3

Cardiopulmonary Bypass: Is MHC Class II expression predictive of outcome?

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3.1 INTRODUCTION

The recognition that surgery and trauma caused a prolonged alteration in monocyte function, with reduced antigen presenting capacity and reduced mitogen-induced synthesis of IL-1 and IFN- γ , occurred as early as 1988 (Faist, Mewes and others, 1988). In the same year, a report appeared showing a close correlation between the failure of peripheral blood monocytes to express HLA-DR by the 7th day after injury, and the development of infection and death in severely injured patients (Hershman, Cheadle and others, 1988). Since this report there have been numerous similar studies showing that persistently reduced HLA-DR expression following surgery or trauma identifies a subgroup of patients at increased risk of infection and poor prognosis (Cheadle, Hershman and others, 1991; Giannoudis, Smith and others, 1999; Hershman, Cheadle and others, 1990; Klava, Windsor and others, 1997; Tschaikowsky, Hedwig-Geissing and others, 2002; van den Berk, Oldenburger and others, 1997; Wakefield, Carey and others, 1993). However the surgical patients reported were general, orthopaedic or adult trauma patients, and there is little research into the effect of cardiac surgery and bypass on MHC Class II expression.

There are three reports showing that cardiac surgery reduces monocyte surface HLA-DR expression, and that the reduction is greater in patients where CPB was utilized (Hiesmayr, Spittler and others, 1999; McBride, Armstrong and others, 1995; Rinder, Mathew and others, 1997). However none of these studies correlate expression with measures of surgical insult, development of sepsis or other outcome measures. One very recent adult study reported a significant reduction in MHC Class II expression in patients undergoing cardiac surgery, but found no predictive value in the level of reduction at 24 hours and the development of sepsis/SIRS (Oczenski, Krenn and others, 2003). However their measurements did not continue beyond 24 hours.

At the initiation of this thesis there was only one study describing the presence of reduced monocyte HLA-DR expression in critically ill children, and no studies on the effect of CPB on monocyte function in the paediatric population (Peters, Petros and others, 1999). This chapter sets out to describe in detail the effect of cardiac surgery involving cardiopulmonary bypass on monocyte MHC Class II surface expression, and

to determine if monocyte deactivation, as indicated by reduced MHC Class II expression, was a factor in the development of sepsis/SIRS following cardiopulmonary bypass.

3.2 METHODS

3.2.1 Patient group

This was a prospective observational study carried out over an 11 month period involving children up to 16 years of age. Patients were excluded if they had a known immunodeficiency, were on immunomodulatory medication, or were pre-term at the time of the operation (< 36 weeks gestation). Children undergoing surgery for hypoplastic left heart syndrome, were also excluded as this condition had a high early complication/death rate not representative of other paediatric cardiac surgery, which may have biased outcome.

All children were elective admissions for cardiac surgery involving CPB. Children were clinically well and believed to be free from infection at the time of surgery. At surgery all children routinely had arterial and central venous catheters placed, and blood sampling for this study was obtained through one of these lines. Depending on the operation undertaken, and the perceived risk of arrhythmias or pulmonary hypotension, some children had additional direct atrial lines and pacing wires placed at the end of surgery.

3.2.2 Anaesthesia and CPB Management

Within the institution where this study took place, there is a team of dedicated cardiac anaesthetists and perfusionists. Whilst the anaesthetic agents used in cardiac surgery vary slightly depending on the child's age and cardiac defect, the pharmacological agents used in theatre can be summarised as follows. Anaesthesia was induced with either intravenous ketamine (2mg/kg), or the inhalational agent sevoflurane in oxygen. During bypass, anaesthesia was maintained with isoflurane and fentanyl, with pancuronium for neuromuscular blockade. Prior to aortic cannulation, 300 IU/kg of

heparin were administered to achieve systemic anticoagulation. The bypass circuit was primed with crystalloid, colloid, mannitol (0.5g/kg), packed red blood cells (150-300ml), heparin (2500 IU/unit packed cells), 8.4% sodium bicarbonate (20 ml/unit packed cells), and calcium chloride (3 mmol/unit packed cells). Hollow fibre membrane oxygenators were used to achieve gas exchange. Mean perfusion pressure during bypass was maintained using non-pulsatile flow with isoflurane to vasodilate and metaraminol to vasoconstrict as required. Full flow on bypass was calculated using a cardiac index of 2.4 l/min/m². Systemic perfusion pressure was maintained at a mean of 35-45mmHg throughout the time the child was on bypass.

After the surgeon determined the desired temperature for operating, core temperature was controlled using a heat exchanger in the bypass circuit and monitored with a nasopharyngeal probe. All operative repairs requiring circulatory arrest were performed at 18°C, and the myocardium was protected with 'St. Thomas #1' crystalloid cardioplegia solution (30 ml/kg). Modified ultrafiltration was utilised at the end of bypass, to raise the haematocrit to 40 in all neonates, and any child who had undergone haemodilution, long bypass time or been cooled. The effects of heparin were reversed at the end of the procedure with protamine (6 mg/kg).

3.2.3 Clinical Care

All patients were admitted to a specialised cardiac intensive care unit (CICU) from theatre, where they were looked after by paediatric cardiac intensivists and senior specialist registrars in conjunction with the cardiothoracic surgeon and consultant cardiologists. Clinical care was unaffected by participation in the study and results of investigation were not available to the clinical team. Operations were grouped according to technical difficulty utilising the classification published by Stark et al. (Stark, Gallivan and others, 2000). The Paediatric Index of Mortality (PIM) (Shann, Pearson and others, 1997) was assessed on admission to the Cardiac Intensive Care (CICU) as an indication of the physiological state of the patient at the end of theatre (Appendix II).

Clinical progress was monitored daily, with duration of ventilation and postoperative complications recorded. Postoperative complications which were marked as present or absent each day included: sepsis, low cardiac output, arrhythmias, ischemic changes on ECG, bleeding, requirement for chest re-exploration, lobar collapse or consolidation, tracheobronchomalacia, and hepatic or renal dysfunction. As per protocol, all children received 24hrs of perioperative antibiotic cover (amikacin and flucloxacillin). Blood cultures were taken at the treating clinicians discretion if there was a clinical concern about sepsis and an empirical antibiotic regimen according to hospital protocol was started (amikacin and teicoplanin).

For the purpose of this study, the following outcome measures were recorded: sepsis, SIRS, length of stay (LOS) in CICU, and death. An ongoing audit (April 1998 –) demonstrated that CICU length of stay is bimodally distributed, with the majority of children staying < 5 days. Short stay was defined as ≤ 5 days on ICU, and long stay as > 5 days. A modification of the consensus conference (1992) definition of sepsis/SIRS was used (Appendix III). In view of the frequency with which these children received ventilation and inotropic support, respiratory and heart rates were felt to be unreliable indicators. Therefore **SIRS** was more tightly defined as:

temperature	>38°C or <36°C (not artificially controlled), and
WCC	>12,000 or <4,000 cells/mm ³ .

Sepsis was defined as positive blood cultures in the setting of SIRS.

3.2.4 Study design

An overview of the study design is given in figure 3.1. Patients were recruited preoperatively and the first blood sample collected after induction of anaesthesia on line insertion. The second sample was taken at the end of surgery following modified ultrafiltration. A third sample was collected in the cardiac intensive care unit 6 hours post surgery. Subsequent samples were collected at 0900 each morning, to avoid any effect of diurnal variations in MHC Class II expression, for the duration of the patient's intensive care stay.

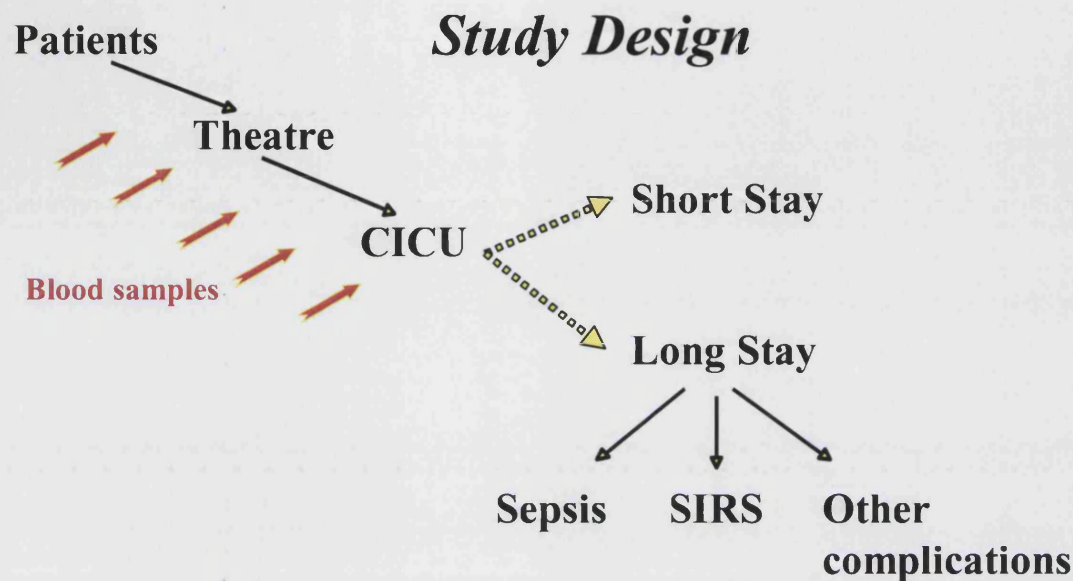


Figure 3.1 An overview of study design.

Patients were consented pre-operatively and the first sample was taken on induction of anaesthesia. Additional samples were drawn following bypass before skin closure, 6 hrs post-operatively, and daily at 0900 throughout the ICU stay. Outcome measures included duration of CICU stay, development of sepsis or SIRS and death.

3.3 STATISTICS

As MHC Class II was expressed as a percentage, and therefore not normally distributed, comparison between groups was performed with the Mann-Whitney test. A 2-tailed t-test for equality of means was utilised when comparing MFI pre-operatively. Exact confidence limits were calculated for proportions with correction for small sample sizes. Multivariable logistic-regression analysis was used to evaluate individual variables and the best combination of variables predicting: prolonged stay; development of sepsis; and sepsis/SIRS.

3.4 RESULTS

Prior to collecting patient samples a series of experiments were undertaken to assess the stability of the staining technique. In view of the range of times and conditions over which blood samples were going to be collected, the effect of delaying the time to staining and time to analysing were examined. In addition the first 20 patients were examined in duplicate to ensure reproducibility of staining.

3.4.1 Reproducibility of Staining

As described in section 2.4 and 2.5 whole blood was collected into anti-coagulant and stained for MHC Class II expression. Analysis of duplicates from the first patients revealed a variability of less than 2% (Fig 3.2). This was independent of the level of MHC Class II as shown in Fig 3.2

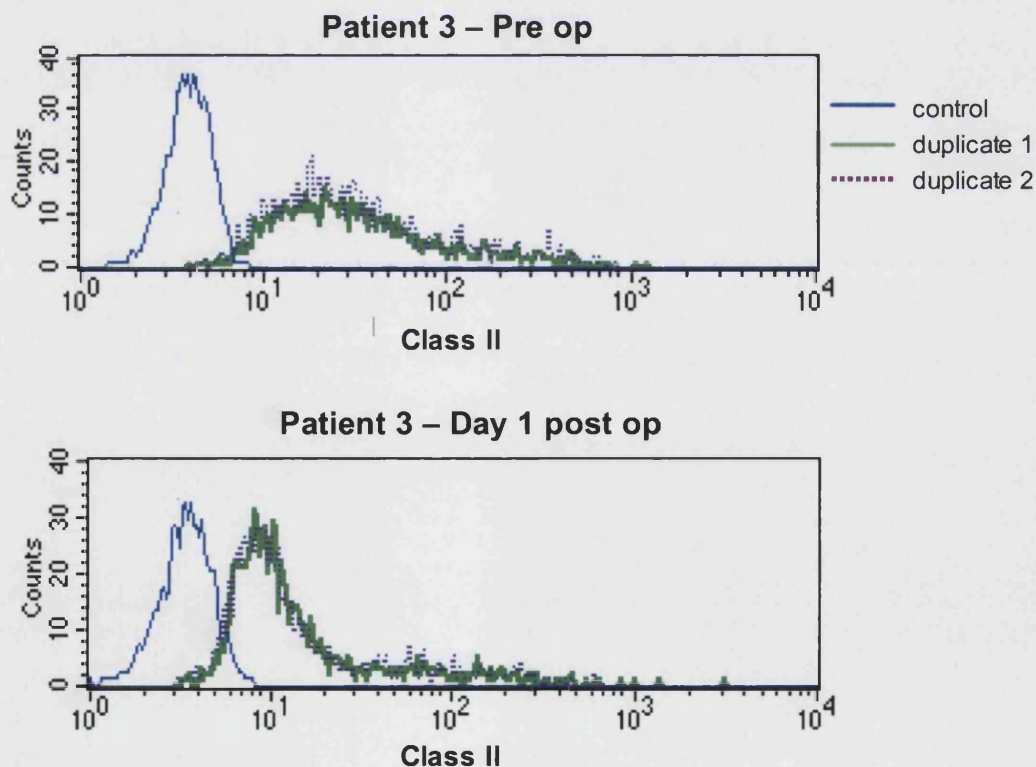


Figure 3.2 Surface staining of monocytes for MHC Class II. Two histograms demonstrate MHC Class II staining pre-operatively and on Day 1 post-operatively for study patient 3. Duplicate staining of initial patient samples showed high reproducibility for the technique used. The marker on each histogram indicates the percentage of monocytes that are staining positive for MHC Class II above the background control staining.

3.4.2 Effect of delay in staining on whole blood MHC Class II expression

Experiments were performed in order to ascertain whether the result obtained for MHC Class II varied if the sample of whole blood was left on the bench or in the fridge in the anaesthetic room prior to staining. Figure 3.3 shows that there was a downward drift in the percentage of monocytes expressing MHC Class II. This reached as much as 10% when anticoagulated whole blood was left at room temperature for 2 hours prior to staining. The reduction in cells expressing MHC Class II was less if the patient sample was placed at 4°C. In contrast, the expression of MHC Class II per cell, as assessed by the median fluorescence intensity, appeared to drift up when there was a delay between collection of the blood sample and staining regardless of whether the sample was left at room temperature or 4°C.

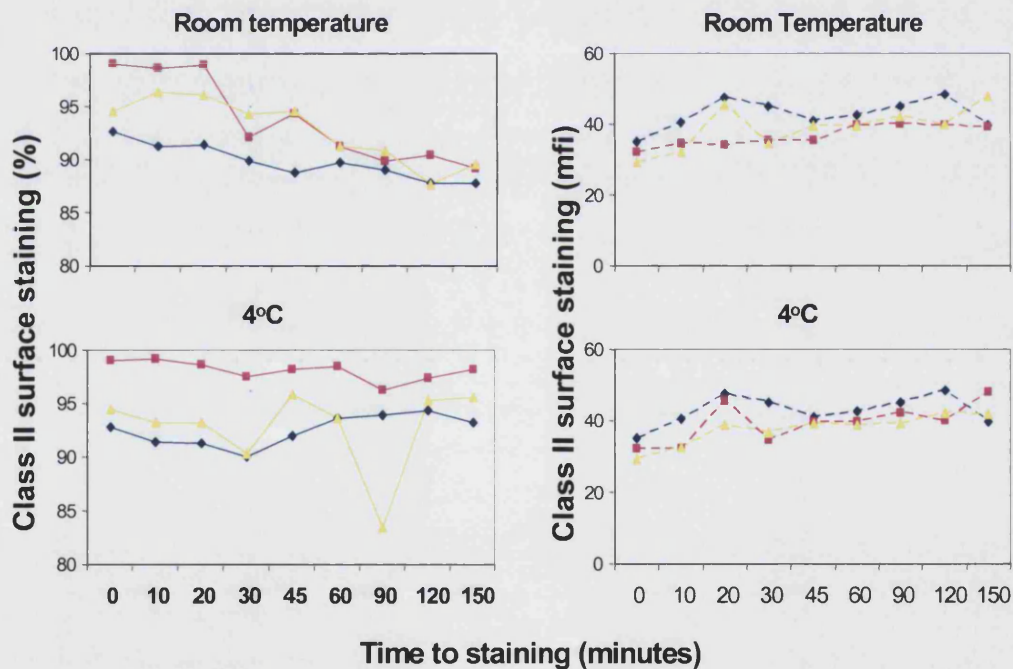


Figure 3.3 The effect of delay in antibody staining of whole blood. Blood drawn from 3 healthy controls was allowed to stand at either room temperature or 4°C for 10-150 minutes before staining for monocyte MHC Class II expression. Results of antibody staining were compared to samples stained immediately. Whole blood samples that were left at room temperature had a reduction of up to 8% in surface staining for MHC Class II expression (%) and a trend upwards in median fluorescence intensity. This was less marked when the samples were placed at 4°C between collection and staining.

3.4.3 Effect of delaying analysis on MHC Class II expression

Preliminary experiments were undertaken to examine the effect of time on the fluorescence of bound antibody. Whole blood from 3 of the initial patients was stained for surface expression of MHC Class II, stored at 4°C and repeatedly analysed over a 7 day period. Analysis of samples showed a marked reduction in surface MHC Class II expression (both % and mfi) from 24 hours after staining (Fig 3.4). Samples analysed within 24 hours of staining showed minimal variation.

Taken together the results from the above experiments indicated that MHC Class II expression should be analysed on samples that are stained within thirty minutes of venesection and analysed within 24 hours.

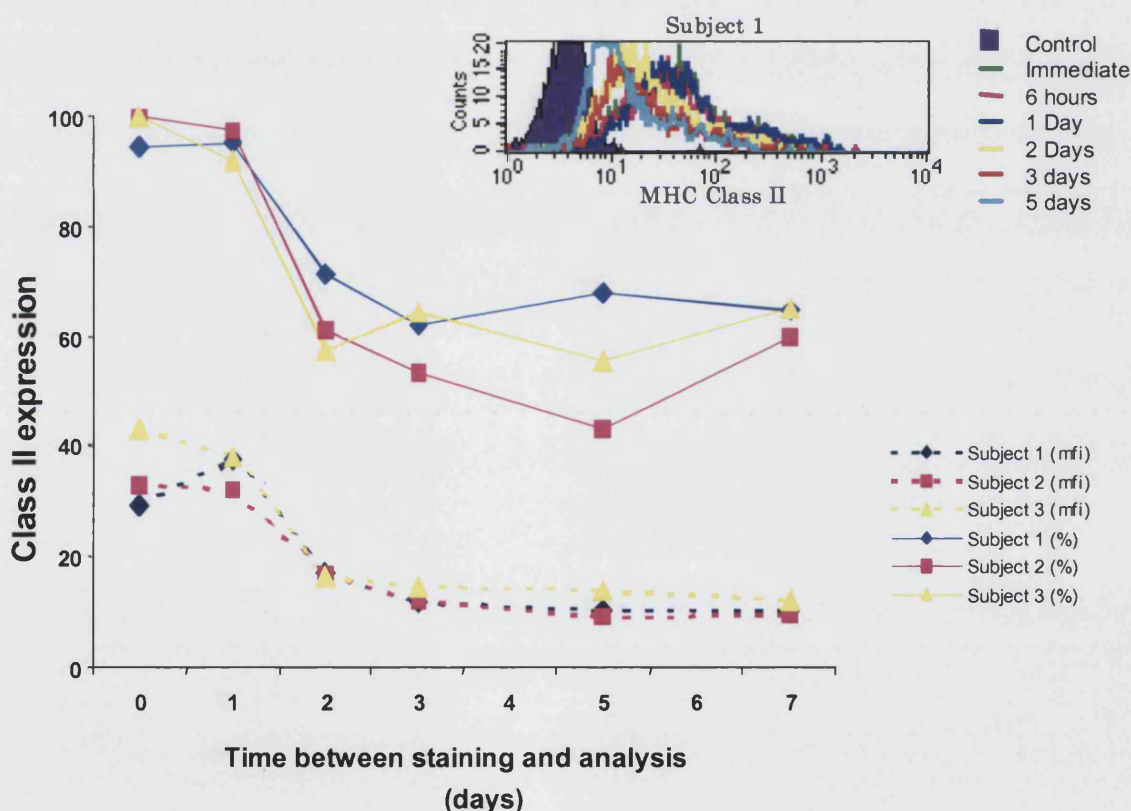


Figure 3.4 Effect of delaying analysis on MHC Class II expression. Samples from three initial patients were repeatedly analysed each day for 7 days to assess the effect on the measurement of MHC Class II expression. Initial staining occurred within 30 minutes of venesection, and samples were fixed and stored at 4°C protected from light between times of analysis. There was little difference in results obtained from samples analysed on the day or 24 hours after staining, but there was a marked reduction in Class II expression if samples were left 48 hours or more before analysis. Inset shows MHC Class II histogram for subject 1.

3.4.4 Patient Characteristics and Operative Details

Once the optimal conditions for MHC Class II staining and analysis had been determined, patient enrolment proceeded. Eighty-two infants and children were studied over the 11 months. An additional five parents were approached but refused participation. The median age of the population studied was 10 months (range 2 days – 16 years). The male:female ratio was 3:2 and the median weight was 8 kg (2 – 50 kg).

A range of operative procedures were performed on these patients. These can be subdivided into the following groups:

relief of right ventricular outflow tract obstruction	(20%),
ventricular or atrial septal defect closure	(16%),
pulmonary artery reconstruction	(12%),
complex shunts including total cavopulmonary connection, arterial or atrial switch, and Glenn procedure	(14%),
complete atrioventricular septal defect repair	(10%),
valve repair / replacement	(10%)
and others	(18%).

Details of cardiac surgery are described in section 3.2.2. In this population, the median duration of CPB and aortic cross-clamp time was 82 min (range 16–305), and 40 min (range 5–178) respectively. Eleven percent (9/82) of the operations were performed under deep hypothermic circulatory arrest. Sixty-one (75%) patients had modified ultrafiltration prior to coming off bypass in an effort to remove excess total body water. Four of the 82 patients had delayed sternal closure, indicating significant local tissue oedema impairing cardiac function. All enrolled patients completed the study protocol.

3.4.5 Outcome

As expected, the intensive care stay (LOS) following surgery was distributed bimodally, with an overall median stay of 3 days (Fig 3.5). In the majority of patients (63%, 52/82) the LOS was ≤ 5 days, with the remainder (37%, 30/82) requiring a median of 10 days (6–50 days). There was no statistically significant difference between LOS in this study

group and the audited population ($p=0.82$). Eleven patients developed proven sepsis and three further patients fulfilled the criteria for SIRS in the postoperative period. Other complications seen included arrhythmias (43%), poor cardiac function requiring inotropic support (23%), pulmonary hypertension (16%), and multiorgan failure (10%). All complications were identified and classified by a single senior clinician as part of the on-going audit programme. Only 2 patients died during this study, both within the long stay group. These data were included.

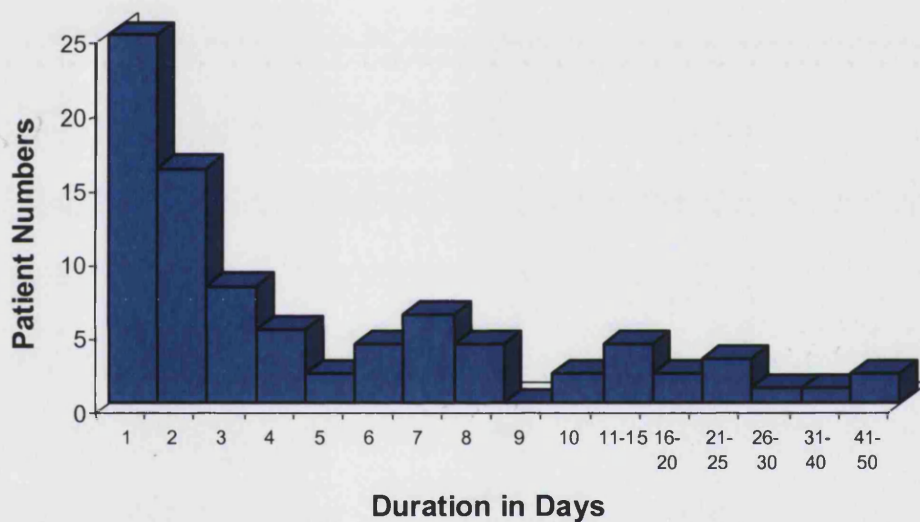


Figure 3.5: Duration of stay in CICU following cardiac bypass surgery.

Short stay was defined for the purpose of this study as ≤ 5 days (median 48 hrs), and long stay as > 5 days (median 10 days). The median length of stay for the whole study population was 3 days.

3.4.6 Monocyte MHC Class II expression in patients undergoing CPB

The expression of MHC Class II on the surface of circulating monocytes was high in all patients pre-operatively. The majority (80%) of patients had MHC Class II expression greater than 95% (Fig 3.6). Following cardiac bypass, MHC Class II expression was

significantly reduced compared to pre-operative levels. Surface expression of MHC Class II remained reduced for several days, with the lowest levels seen at 48-72hours ($p < 0.01$).

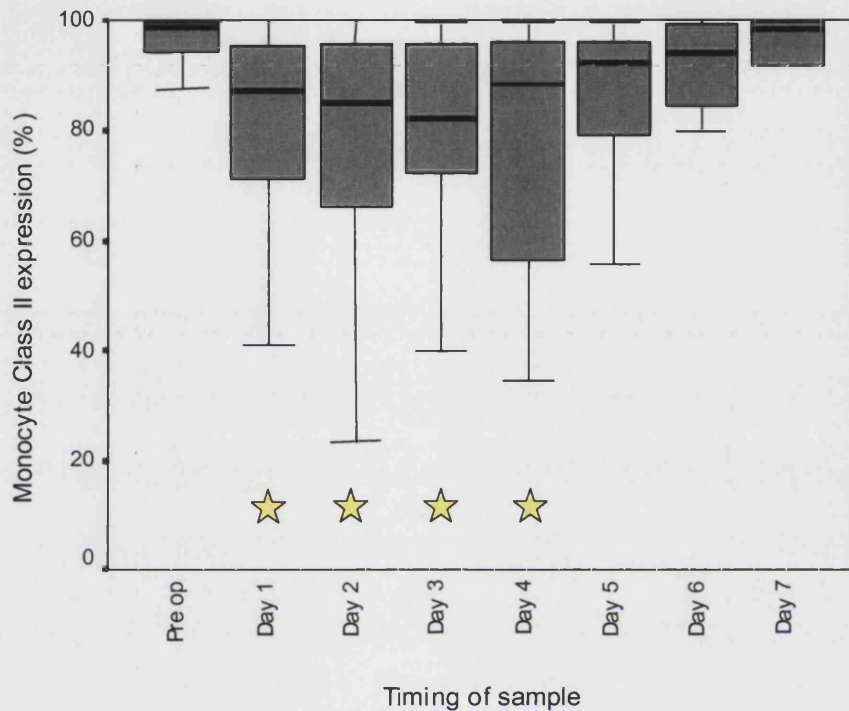


Figure 3.6 Monocyte MHC Class II expression in all patients following cardiopulmonary bypass. Blood was taken from each study patient at anaesthetic induction (pre op), and at 9 am each day following the operation. Data are shown in *box and whiskers* plots, indicating the median, IQR and range. There was a significant reduction (★ Mann-Whitney $p < 0.01$) in monocyte MHC Class II expression (HLA-DR) for the first 4 post operative days, compared with pre operative expression.

3.4.7 Monocyte MHC Class II expression related to Morbidity: Length of Stay

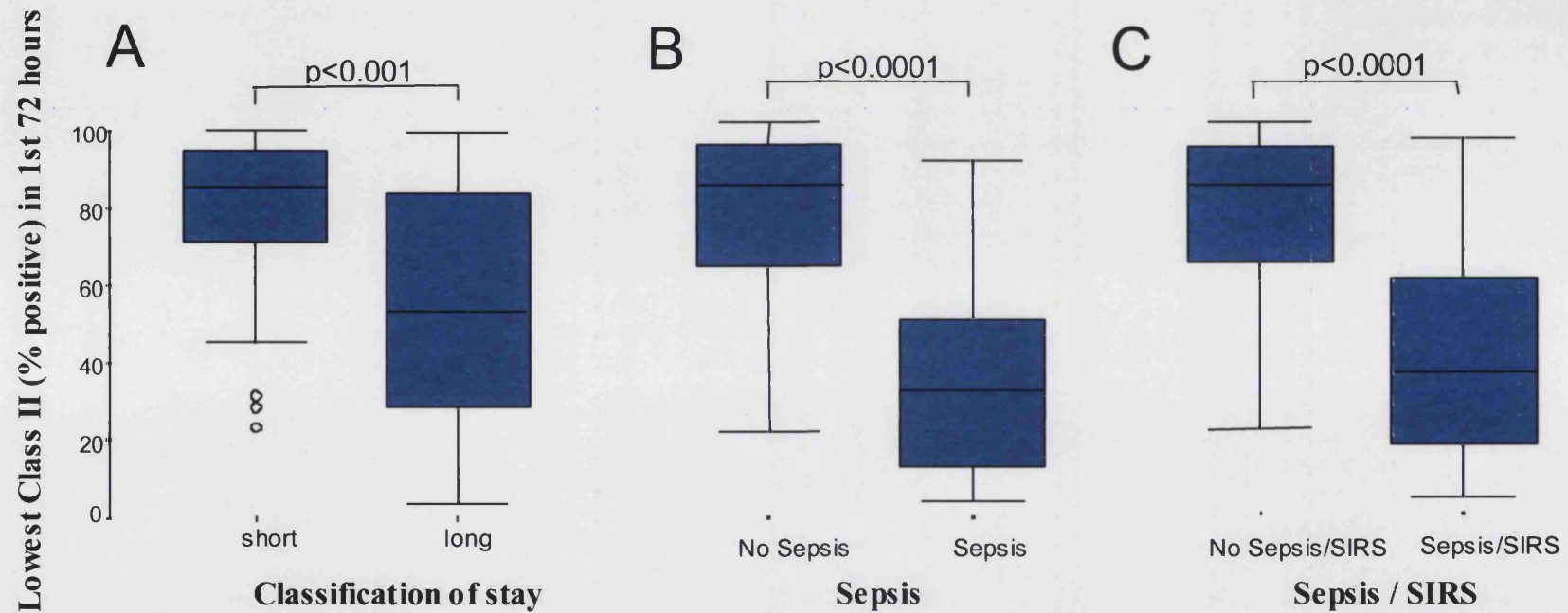
In order to address the question of whether this early reduction in surface expression of MHC Class II was related to patient recovery, the MHC Class II levels were compared with length of stay. The nadir of MHC Class II expression in the first 72 hours post

bypass was significantly lower in long stay (>5 days) as compared to the short stay patient group (≤ 5 days); median 53% vs. 87% (Mann-Whitney $p < 0.001$) (Fig 3.7a). That is, those patients that subsequently required prolonged intensive care support had a far greater reduction in their MHC Class II expression in response to cardiac surgery.

3.4.8 Monocyte MHC Class II expression related to Morbidity: Development of Sepsis / SIRS

If the reduction in MHC Class II expression in the early post-operative period directly affected the patients recovery, one possible means may have been through increased susceptibility to infection. The eleven patients who developed proven sepsis all required a prolonged stay in Intensive care. Three further patients, who were classified as SIRS, also required a prolonged stay. While no patients fulfilled the criteria for sepsis/SIRS in the first 72 hours post-operatively, the nadir of MHC Class II in this period was significantly lower in patients that went on to develop sepsis/SIRS. Those patients who subsequently developed sepsis/SIRS had a marked reduction in the expression of MHC Class II on circulating monocytes (<30%) in the early post-operative period. This compared to 84.7% of monocytes expressing MHC Class II in the patient group that remained non-septic ($p < 0.0001$). This reduction in MHC Class II expression was only slightly less and still highly significant, when patients classified as SIRS were considered together with sepsis: median 32% vs 85% ($p < 0.0001$) (Fig 3.7b&c).

It is important to note that the reduction in surface MHC Class II expression preceded the clinical development of both sepsis and SIRS, with the median interval between the lowest value of MHC Class II and development of sepsis being 5 days (IQR 4-12).



3.4.9 Surgical insult and the reduction in postoperative monocyte MHC Class II expression

The most obvious question is whether the reduction in post-operative MHC Class II expression is merely reflective of the degree of surgical insult, and therefore at a greater risk of post-operative complications. MHC Class II expression in the first 72 hours post-operatively did **not** correlate with any of the markers of the severity of insult (Table 3.2). Nor did it correlate with age or the physiological state of the patient on arrival to ICU. In addition, post-operative MHC Class II expression was unrelated to the identity of the surgeon (Kruskal Wallis $p=0.57$).

	Lowest MHC Class II expression in first 72 hours post-op	
	Correlation coefficient	Significance (p)
Age	-0.04	0.71
Bypass time	-0.09	0.43
Cross-clamp time	-0.14	0.22
Lowest temperature	0.20	0.09
Operation severity group	-0.06	0.62
PIM	-0.16	0.26

Table 3.1 Correlation between MHC Class II expression post-operatively and age, measures of surgical insult, and physiological state of the patient on return to ICU from theatre (PIM). There was no correlation between the lowest recorded level of MHC Class II expression on monocytes post-operatively and measures of surgical insult.

3.4.10 Post-operative outcome was related to surgical insult

Whilst the observed reduction in MHC Class II expression did not appear to be directly related to the severity of the surgical insult, the surgery performed did influence patient post-operative recovery. In table 3.3 indicators of the complexity of the surgery (operation group, bypass time, and cross-clamp time) and the physiological state on arrival on ICU (PIM risk of mortality) for the patient group studied, are shown. Patients who developed sepsis, (or sepsis/SIRS) or required >5 days ICU care had longer, more complex procedures and greater physiological derangement on return to the ICU.

	Stay			Sepsis			Sepsis / SIRS		
	Short <i>n</i> =52	Long <i>n</i> =30	<i>p</i> value	no <i>n</i> =71	yes <i>n</i> =11	<i>p</i> value	no <i>n</i> =68	yes <i>n</i> =14	<i>p</i> value
Age	0.96 (0.32-5)	0.63 (0.15-3.3)	0.29	0.75 (0.27-4.5)	1.79 (0.23-13.8)	0.47	0.78 (0.26-4.6)	1.04 (0.3-12)	0.5
Operation group	2 (1-2)	2 (2-3)	0.08	2 (1-2)	2.5 (2-3)	0.005	2 (1-2)	2 (2-3)	0.02
Bypass time	62 (33-92)	87 (69-133)	0.007	69 (34-97)	112 (85-145)	0.004	69 (34-98)	108 (81-141)	0.01
X-clamp time	22.5 (0-41)	43 (13-64)	0.021	27 (0-46)	48 (20-67)	0.08	26 (0-46)	65 (23-66)	0.09
Risk of Mortality (PIM)	0.06 (0.05-0.1)	0.09 (0.06-0.11)	0.042	0.07 (0.05-0.1)	0.11 (0.09-0.13)	0.02	0.07 (0.05-0.1)	0.11 (0.08-0.13)	0.02

Table 3.2 Age, measures of the perioperative course and severity of physiological derangement at ICU admission between outcome groups. Median values and interquartile ranges (IQR) are shown. Comparison are made with the Mann-Whitney tests. Higher PIM score and bypass times were seen in the long stay patients and in those that developed sepsis or SIRS.

3.4.11 Reduced monocyte MHC Class II expression remains strongly associated with the development of sepsis, sepsis/SIRS, and prolonged requirement for intensive care after correcting for other factors.

So the question arises, what role does reduced monocyte MHC Class II expression play in the morbidity observed following cardiac surgery involving CPB? To assess the contribution of MHC Class II to outcome, 3 logistic regression models were constructed to identify the factors that were independently associated with a requirement for (i) prolonged ICU stay, (ii) development of sepsis, and (iii) development of sepsis/SIRS (Table 3.4 & 3.5). MHC Class II remained highly significantly associated with a requirement for increased stay, development of sepsis, or sepsis/SIRS ($p < 0.001$), after correction for all covariates (age, sex, operation group, bypass time, surgeon, PIM) regardless of the method of variable entry. Whilst univariate analysis showed that bypass time was associated with these outcomes, this effect was no longer apparent after correction for the MHC Class II expression. This indicates that monocyte MHC Class II expression was independently associated with the subsequent development of sepsis or sepsis/SIRS.

Because length of stay is a continuous measure, and the division of length of stay into short (≤ 5 days) and long stay (> 5 days) is a somewhat arbitrary one, further regression models were constructed utilising length of stay as the continuous dependant variable. The monocyte Class II expression remained independently associated with length of stay after correction for all of the above covariates ($p < 0.001$). (Results not shown)

3.4.12 Reduced monocyte MHC Class II expression predicts the development of post-operative sepsis/SIRS.

Finally, the performance of reduced MHC Class II expression as a predictor of the development of sepsis, sepsis/SIRS or prolonged ICU stay was examined. The receiver operating characteristic (ROC) curve (Hanley and McNeil, 1982; Hanley and McNeil, 1983) examines the probability that MHC Class II expression on monocytes will correctly predict those patients most likely to develop complications. This analysis addresses the performance of MHC Class II across all values. The ROC curve for

	Stay			Sepsis			Sepsis / SIRS		
	Signif.	O.R	95% C.I	Signif.	O.R	95% C.I	Signif.	O.R	95% C.I
Age	0.81	1.01	(0.92-1.12)	0.30	1.07	(0.94-1.21)	0.16	1.09	(0.97-1.22)
Operation group	0.14	1.60	(0.85-3.01)	0.01	4.13	(1.40-12.2)	0.09	2.07	(0.90-4.77)
Bypass time	0.13	1.01	(1.00-1.02)	0.02	1.01	(1.00-1.02)	0.04	1.01	(1.0-1.02)
X-clamp time	0.04	1.02	(1.00-1.03)	0.22	1.01	(0.99-1.02)	0.42	1.01	(0.99-1.02)
Risk of Mortality (PIM)	0.94	0.89	(0.05-15.12)	0.81	0.96	(0.64-1.40)	0.46	0.47	(0-153.7)
Lowest MHC Class II expression in 1 st 72hrs	0.0003	0.96	(0.94-0.98)	0.0003	0.94	(0.92-0.97)	0.0002	0.95	(0.93-0.98)

Table 3.3 Univariate logistic regression analyses were performed to assess the contribution of each variable to the 3 outcome measures of stay, sepsis, and sepsis/SIRS. Whilst bypass time significantly contributed to the likelihood of developing sepsis or sepsis/SIRS, it was not significantly associated with a prolonged stay in Intensive Care. The lower the MHC Class II expression recorded in the first 72 hours post operatively, the more likely the child was to require prolonged stay, and the greater the risk of developing sepsis or SIRS. A significance level of < 0.15 was chosen to determine which variable would be analysed in the multivariate analysis below.

	Stay		Sepsis		Sepsis / SIRS	
	Signif.	O.R (95% C.I)	Signif.	O.R (95% C.I)	Signif.	O.R (95% C.I)
Operation Group	0.57	1.26 (0.56-2.78)	0.07	3.73 (0.89-15.7)	0.53	1.4 (0.49-3.97)
Bypass time	0.069	1.01 (1.00-1.02)	0.21	1.01 (1.0-1.02)	0.17	1.01 (1.0-1.02)
Lowest MHC Class II expression in 1st 72hrs	0.008	0.96 (0.94-0.98)	0.0007	0.94 (0.90-0.97)	0.0003	0.95 (0.93-0.98)

Table 3.4 Multivariate logistic regression analysis reveals that low Class II expression remains highly significantly associated with requirement for prolonged stay, development of sepsis or SIRS, after correction for other covariants.

Variables with a significance of < 0.15 on univariate analysis were combined in a logistic regression model in order to control for multiple influences.

This analysis addresses the performance of MHC Class II across all values. The ROC curve for prediction of requirement for 'long stay', subsequent development of sepsis or sepsis/SIRS by the lowest value of MHC Class II expression in the first 72 hours is shown in figure 3.8. The area under the ROC curve is greatest for prediction of the development of sepsis/SIRS at 0.85.

For MHC class II expression to be useful for the prediction of patients at risk from sepsis/SIRS in the clinical setting, a value/threshold with an associated risk is required. Utilising the data presented in figure 3.9 it was possible to calculate a threshold of MHC Class II expression that enabled good prediction of increased risk of sepsis/SIRS. When MHC Class II fell below 60% in the first 72 hours there was a 7.6 fold risk for prolonged ICU stay (95% ci 2.5-23.1). The risk associated with the development of sepsis or sepsis/SIRS was even greater at 11.9 (2.8-51.4) and 12.9 (3.4-47.5) respectively.

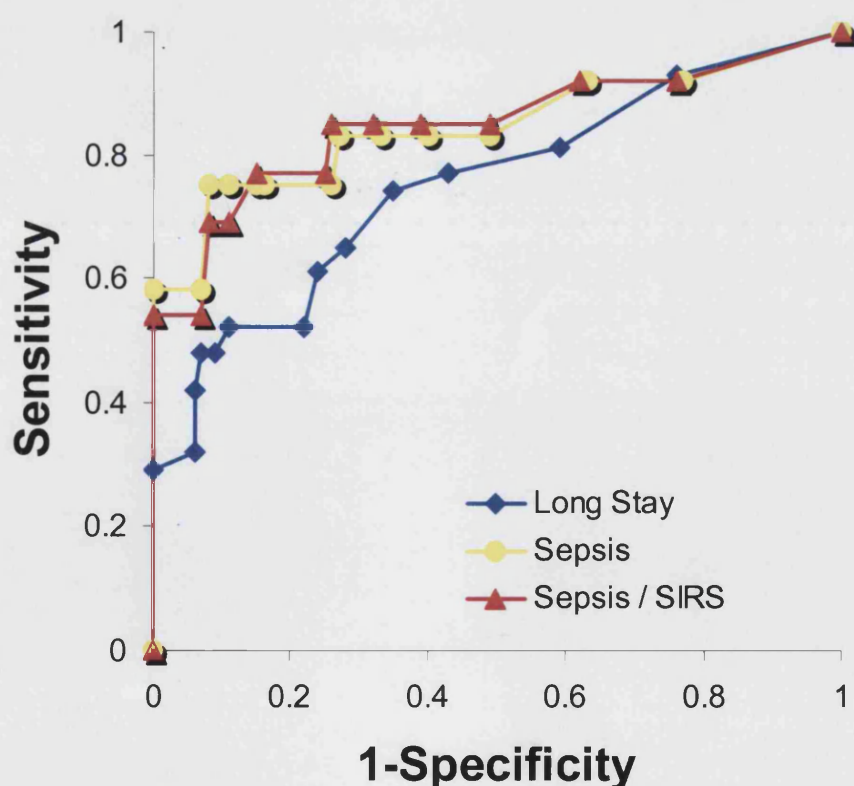


Figure 3.8 Monocyte MHC Class II expression as a predictor of long stay, sepsis, or sepsis / SIRS. The area under the receiver operating characteristic curve for MHC Class II as a predictor of long stay is 0.75. This increases to 0.84 and 0.85 for sepsis and sepsis/SIRS respectively.

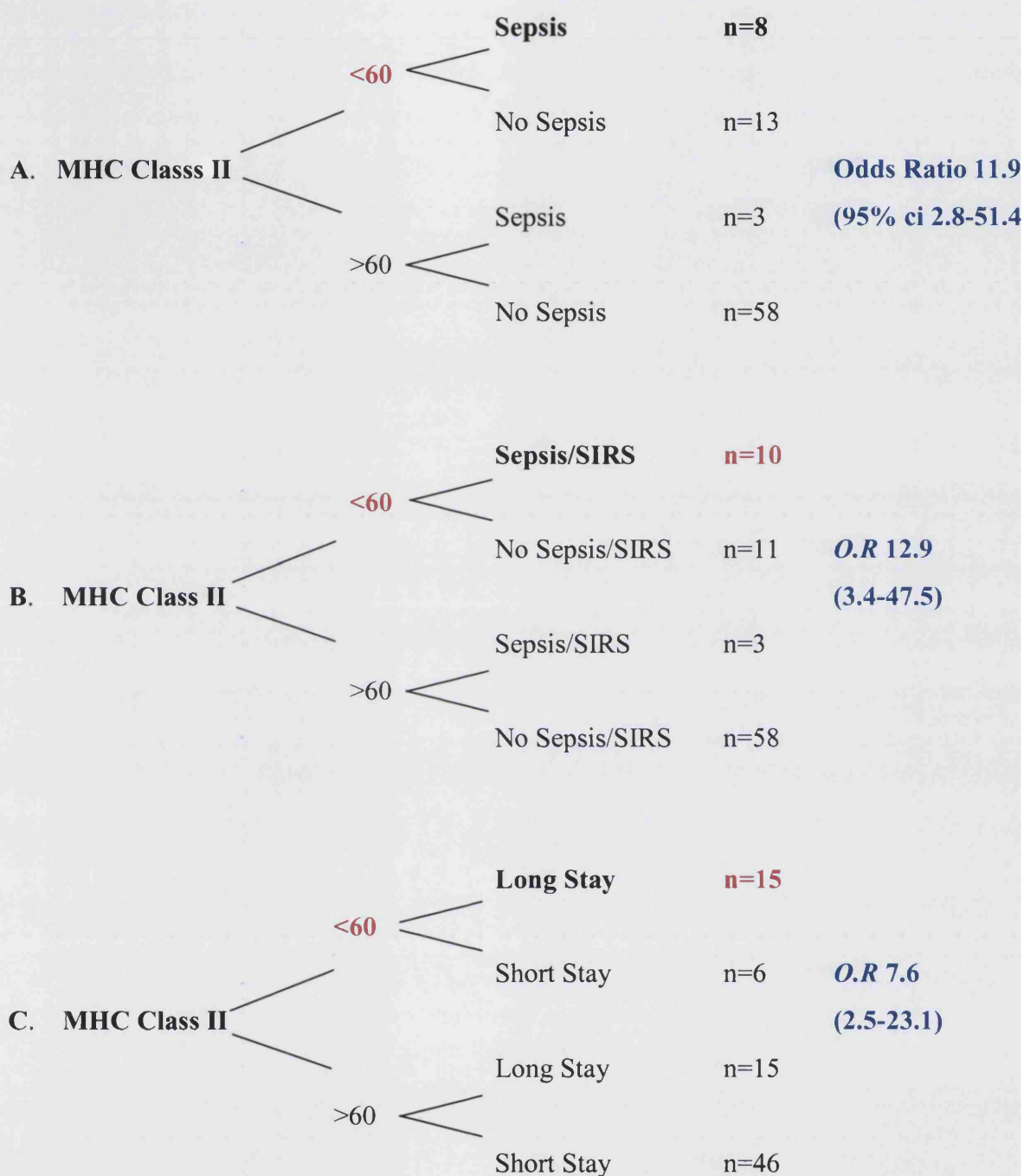


Figure 3.9 Relationship between the lowest postoperative level of Class II expression and the three primary outcome measures of sepsis, sepsis/SIRS and long stay. Flow charts of the proportion of cases classified with sepsis (A), sepsis/SIRS (B) or long stay on intensive care (>5days) (C) are shown according to monocyte MHC Class II expression in the first 72 hours. *O.R.*, odds ratio. At a threshold value of a minimum MHC Class II expression of <60%, highly significant increased odds of these complications were observed.

3.4.13 Monocyte MHC Class II expression in the pre-operative period

The data above indicated that in all patients there was a reduction in MHC Class II in response to surgical stress. However there was marked variability in the MHC Class II expression in response to surgery which could not be explained by the severity or type of surgical insult alone. This raises the possibility that other factors such as the host's capacity to respond to the surgical insult or the inflammatory/immune status of the patient prior to undergoing surgery could be important. To explore this idea, the MHC Class II expression prior to surgery was examined in detailed

There was no significant difference in the percent of monocytes expressing MHC Class II above background pre-operatively between patients who subsequently required short or long stay (median 99 vs. 99.5, $p=0.66$). However, when the intensity of MHC Class II expression (median fluorescence intensity, mfi) on each monocyte was examined, this was significantly lower in cases who subsequently required long stay (long stay vs short stay, $p<0.01$) or developed sepsis ($p<0.001$) or sepsis / SIRS ($p<0.001$) (Fig 3.10).

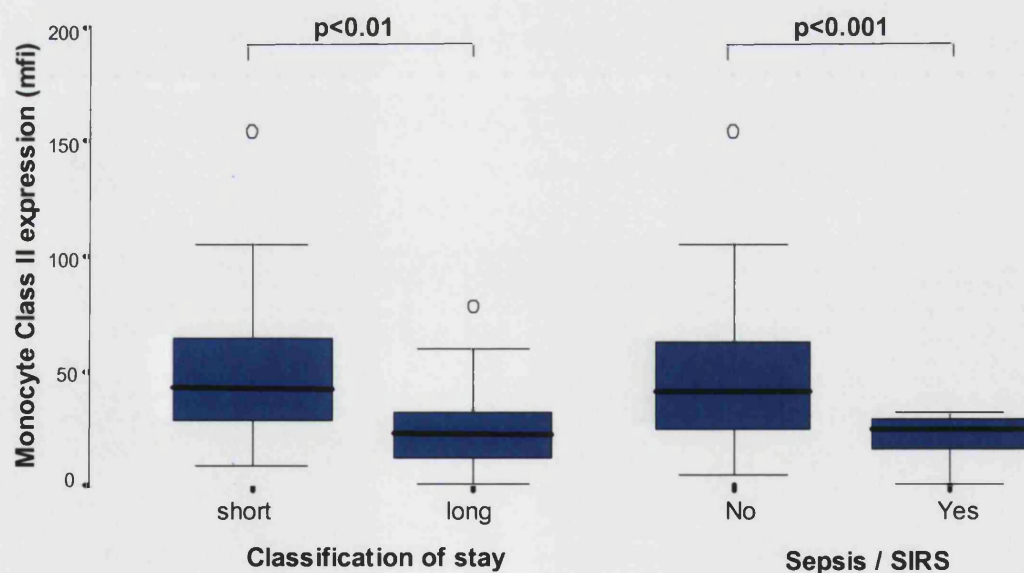


Figure 3.10 Monocyte MHC Class II expression (mfi) pre-operatively. The median fluorescent intensity of MHC Class II on the surface of monocytes was significantly lower pre-operatively in those patients who subsequently required prolonged intensive care support ($p<0.01$), developed sepsis, or sepsis/SIRS ($p<0.001$).

This variability in MHC Class II expression was not a function of age (Fig 3.11).

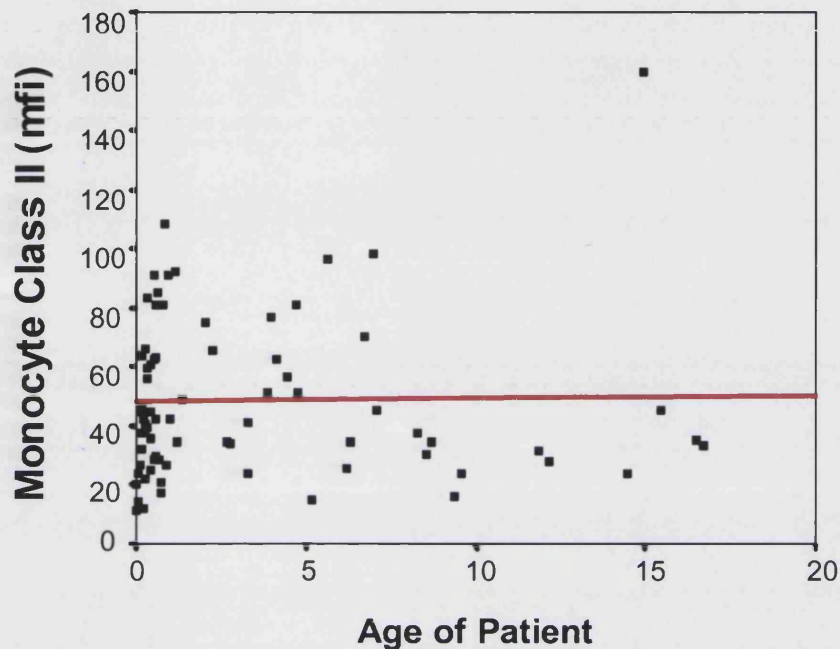


Figure 3.11 Scatter plot of pre-operative MHC Class II expression on monocytes (mfi) against age. The intensity of Class II staining on each monocyte (mfi) pre-operatively is plotted against age at time of surgery. The solid red line show a clear lack of correlation between these two variables.

3.4.14 Reduction in MHC Class II surface expression represents a reduction in HLA-DR surface expression

There are 3 human MHC Class II molecules expressed on the surface of human cells, HLA-DP, -DQ, and -DR. The most abundant of these is HLA-DR. Whilst many clinical studies refer to alterations in monocyte HLA-DR expression, some of these studies use a pan-MHC Class II monoclonal antibody, staining for HLA-DR, -DP, and -DQ (Fumeaux and Pugin, 2002; Giannoudis, Smith and others, 1999; Klava, Windsor and others, 1997; Peters, Petros and others, 1999). In order to confirm that the reduction in MHC Class II expression obtained in this work predominantly represented a reduction in surface expression of HLA-DR, a dual staining technique was utilised. A

subgroup of 10 patients had whole blood stained with a pan -MHC Class II antibody and a specific monoclonal antibody to HLA-DR. The reduction seen in HLA-DR expression over the first 72 hours post operatively paralleled the reduction in surface expression of Class II (both % and mfi) seen in all patients examined (Fig 3.12 and 3.13). Therefore for the duration of this work, the changes seen in the levels of expression surface MHC Class II can be assumed to also represent changes in surface expression of HLA-DR.

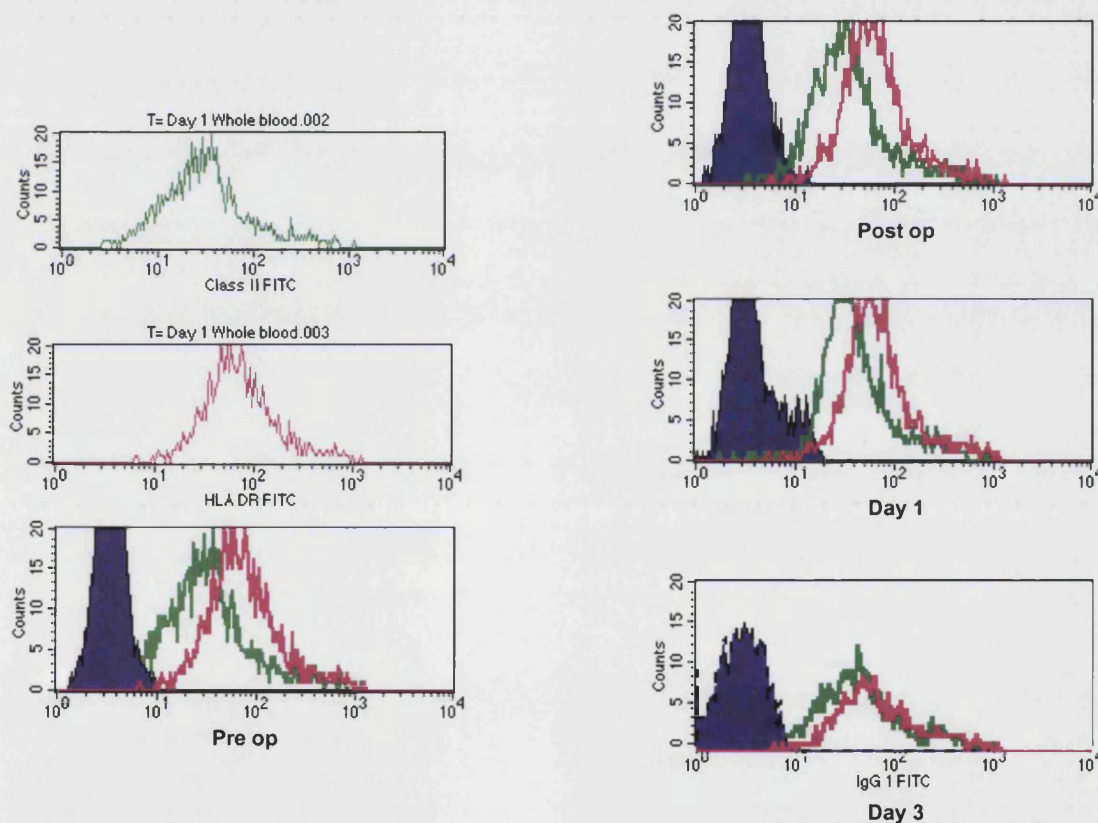


Figure 3.12 Co-staining of samples for both MHC Class II and HLA-DR. A subgroup of 10 patients samples were stained with both MHC Class II – and monoclonal antibody to HLA-DR –. When superimposed, changes seen in Class II expression (% and mfi) were paralleled by the same changes in HLA-DR expression

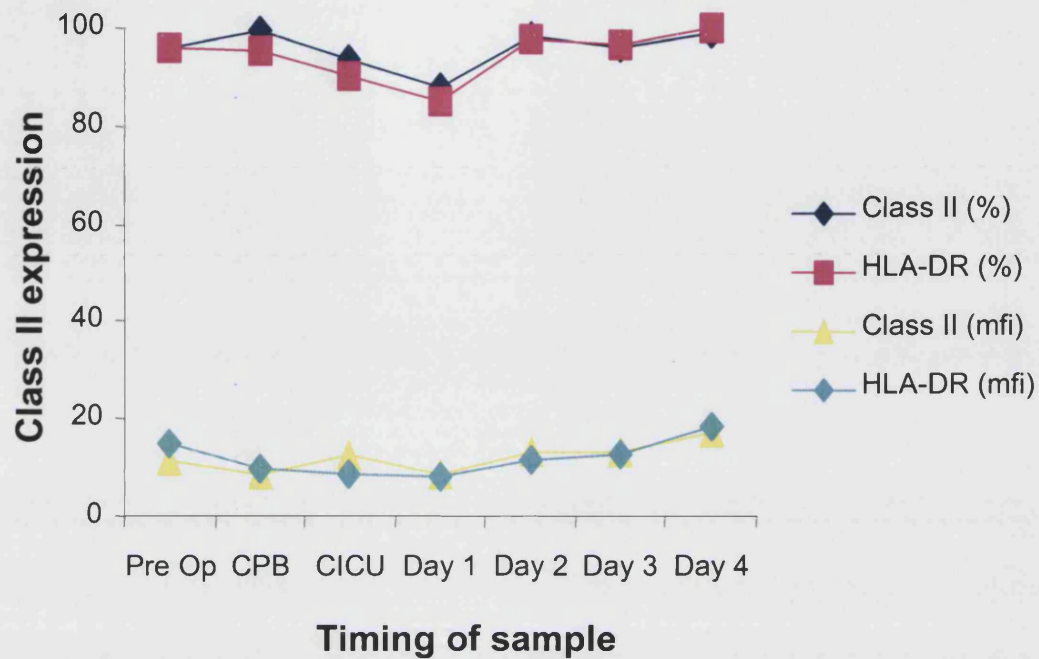


Figure 3.13: HLA-DR expression as compared to Class II expression on whole blood flow cytometry (single patient result shown).

In order to ascertain whether the reduction seen in Class II surface expression was due to a reduction in HLA-DR expression, a subgroup of patients were analysed using monoclonal antibodies to both MHC Class II and HLA-DR. The changes seen on flow cytometry were similar whether whole blood was stained using the pan-MHC Class II antibody or the antibody specific to HLA-DR

3.5 DISCUSSION

This study is the first to describe the expression of monocyte MHC Class II in children undergoing cardiopulmonary bypass. All children showed a fall in monocyte MHC Class II expression following surgery, but levels were lowest in cases that subsequently required prolonged intensive care support, predominantly due to an increased incidence of sepsis (or SIRS). A low level of MHC Class II within the first 72 hours was an independent predictor of the development of sepsis a minimum of 4 days later.

To date the majority of studies in this field have been performed in adults. McBride et al (1995) reported a reduction in MHC Class II expression in adults undergoing CPB ten minutes after induction of anesthesia, with the lowest levels (a reduction of 40%) 2 hours post bypass (McBride, Armstrong and others, 1995). In the results presented above, minimum values for MHC Class II were observed over the first 24 to 72 hrs, which contrasts with the earlier nadir seen in adult patients. Whilst there are now many studies confirming that an inflammatory insult results in a reduction of surface MHC Class II on monocytes, there is conflicting evidence as to whether the Class II expression is predictive of clinical outcome. In two recent adult studies, Perry et al (2003), and Oczenski et al (2003) found that low monocyte Class II expression did not serve as a predictive parameter for the incidence of sepsis and/or as a prognostic parameter for clinical outcome (Oczenski, Krenn and others, 2003; Perry, Mostafa and others, 2003).

There are a number of reasons for possible conflicting results. Oczenski et al (2003) found monocyte HLA-DR fell significantly in all adult patients following cardiac surgery, but that the decrease in levels over the first 24 postoperative hours was not associated with a higher incidence of postoperative sepsis/SIRS, or increased length of stay. This is a short period of monitoring. Results presented in this chapter showed a strong association between MHC Class II expression over the first 72 hours and subsequent complications. In the papers by Hershman, Haveman, Döcke, Volk and Monnerat it was the persistence of low MHC Class II expression following an inflammatory insult (5 days to 3 weeks) that correlated with sepsis and a poor prognosis (Döcke, Randow and others, 1997; Haveman, van den Berg and others, 1999; Hershman,

Cheadle and others, 1990; Monneret, Elmenkouri and others, 2002; Volk, Reinke and others, 1996; Volk, Thieme and others, 1991). Oczenski's apparent conflicting results may just have been due to the study design, where a sustained decrease in MHC Class II expression would have been missed.

Perry's study, which also failed to show MHC class II as a useful predictor of outcome, examined HLA-DR expression on whole blood from septic patients. She looked daily for the first 3 days following admission to ICU. However there was no attempt to correct for duration of illness and therefore phase of inflammatory response. Nor was the persistence of reduced MHC Class II expression examined as a predictor of outcome.

While there are many other reasons why the data presented in this chapter may differ from other studies, including different methodologies, variation in sample handling and timing between staining and analysis, there is increasing evidence to support the findings presented here. There are now more than 10 papers published that support the findings that decreased MHC Class II expression on peripheral blood monocytes is associated with increased risk of infective complications, and an equal number showing that the degree of expression of MHC Class II in patients with established sepsis can be of prognostic significance.

The reasons why MHC Class II expression is associated with the subsequent development of sepsis is unclear. MHC Class II could be reflecting the complexity of the surgery, a prolonged bypass time or deranged post-operative physiology (e.g. low cardiac output). However whilst all of these were associated with the development of sepsis they did not correlate with MHC Class II expression using logistic regression models. Indeed the independence of the relationship between MHC Class II and sepsis, after correction for bypass time and severity of illness (PIM score) on admission, argues against MHC Class II being a non-specific marker of poor perfusion or a difficult intra-operative course. However only more detailed studies looking at cardiac dysfunction in relation to MHC Class II expression can address this issue with some certainty (see Chapter 4).

It is also possible that low MHC Class II expression was indicative of pre-clinical sepsis. However the longish interval between the lowest MHC Class II and the diagnosis of sepsis (median time =5 days; IQR 4- 12 days) argues against this proposition.

As described in Chapter 1, Class II is a component of the major histocompatibility complex (MHC) which is central to the process of antigen presentation. The level of monocyte MHC Class II expression may reflect local concentrations of cytokines. MHC Class II may represent one of the mechanisms by which an initial inflammatory insult (in this case CPB) attenuates the subsequent pro-inflammatory response to a second insult, so called *immune paresis* or *inflammatory stimuli-induced anergy* (Docke, Randow and others, 1997; Volk, Reinke and others, 1996). The mechanisms involved in the development of *immune paresis* are not fully understood. However, recent data suggesting that MHC Class II may have a direct role in mediating the cellular response to endotoxin (Piani, Hossle and others, 2000) may be particularly pertinent. Other mechanisms that may contribute to *immune paresis* include the balance of Interleukin-1 and Interleukin-1 receptor antagonist, NF- κ B and heat shock factor (HSF) at the intracellular level (Adrie and Pinsky, 2000).

In this study, the high rate of sepsis/SIRS may be due to exposure of severely immunoparalysed children to nosocomial pathogens (this is addressed in more detail in Chapter 5). *Immune paresis* has been proposed to be an adaptive self-protective mechanism to prevent the host from excessive pro-inflammatory immune stimulation. However, while this may be of benefit in many scenarios, in a postoperative intensive care patient this may render the host susceptible to nosocomial sepsis. As such, it could be argued that pro-inflammatory stimulation could be of benefit to such individuals. Support for this is provided by a small unblinded study in adult septic patients which suggested that restoration of MHC Class II expression by the administration of Interferon- γ was associated with clinical improvement (Docke, Randow and others, 1997).

This study provides evidence that sub group of patients have reduced low MHC Class II expression and have a 13 fold higher risk of sepsis or systemic inflammation following cardiac surgery with bypass. These individuals can be identified within 72 hours of

surgery and a minimum of four days prior to the onset of sepsis/SIRS. The observation that there was a small but significant difference in Class II expression in the pre-operative samples of patients who subsequently developed sepsis or SIRS suggests the potential of a genetic/other predisposition to developing sepsis/SIRS. This will be examined further in Chapter 6.

CHAPTER 4

Cardiac function and the Inflammatory response to Cardiopulmonary Bypass

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4.1 INTRODUCTION

Chapter 3 demonstrated that cardiac surgery utilising cardiopulmonary bypass was associated with a significant reduction in the surface expression of MHC Class II on peripheral blood monocytes. The study did not address why cardiac surgery was responsible for the reduction in MHC Class II. It did however explore the possibility of whether particular features of the surgery, such as bypass time, aortic cross-clamp time, temperature, and nature of the cardiac repair, were responsible for the change in MHC Class II expression. They all proved to be unrelated to the degree of fall in surface expression. This raises the question as to why cardiac surgery led to a reduction in MHC Class II.

Cardiac surgery is invariably associated with a release of both pro- and anti-inflammatory mediators (Chapter 1 section 1.2.4), and it is possible that the reduction in MHC Class II expression was due to this cytokine response. *In vitro* studies have shown that MHC class II expression is sensitive to some of the mediators seen during or following cardiac surgery. As the cytokine response is believed to reflect the extent of tissue damage, with the degree of release of pro-inflammatory cytokines directly related to the duration of bypass and ischemia (Grunenfelder, Zund and others, 2000;Neuhof, Wendling and others, 2001;Wan, LeClerc, and Vincent, 1997;Wan, Marchant and others, 1996), it is perhaps surprising that MHC Class II expression did not correlate with markers of surgical insult (bypass time, cross-clamp time, operating temperature, operating group). One explanation is that the indices used were poor surrogates for the surgical insult. Some authorities believe that a better correlate of the host response to the surgery is early post-operative cardiac function.

In 1995, Wernovsky published a landmark paper in which he described for the first time the detailed haemodynamic profile and post-operative course of 171 neonates and infants following an arterial switch procedure (Wernovsky, Wypij and others, 1995). In this paper, he was able to show that the nadir in cardiac index occurred 9-12 hours post release of the aortic cross clamp. The reasons for this nadir are unknown but were suggested to be due to pro-inflammatory mediators (Cain, Meldrum and others, 1999;Hovels-Gurich, Vazquez-Jimenez and others, 2002;Wan, DeSmet and others, 1996;Wan and Yim, 1999;Wernovsky, Wypij and others, 1995). Whilst there have

been several studies describing various aspects of the inflammatory response to CPB in children, there is no detailed description of cytokine release over the first 24 hours and how this relates to cardiac function.

This chapter sets out to describe in detail the pro and anti-inflammatory cytokine response to cardiac surgery involving CPB over the first 24 post-operative hours, and to test the following hypotheses:

- (i) the nadir in cardiac function seen during the first post-operative night is due to the pro-inflammatory response to bypass.
- (ii) the reduction in surface expression of Class II on monocytes in the early post-operative period is a reflection of the cytokine balance arising from the inflammatory insult.

4.2 METHODS

4.2.1 Patient Selection

This prospective observational study was conducted over a 15-month period, involving thirty-nine infants and children up to 24 months of age undergoing cardiac surgery involving cardiopulmonary bypass. Children were excluded if they were neonates <36 weeks gestation, were less than 2500gm, or had known immunodeficiencies. This was part of a collaborative study undertaken with the Cardiothoracic Surgeons at Great Ormond Street Hospital for Sick Children, NHS Trust.

4.2.2 Anaesthesia and CPB management

Anaesthesia and cardiopulmonary bypass was carried out as described in section 3.2.2, except that either blood or cold crystalloid cardioplegia (St. Thomas' #1 solution) was used dependant on the operator's preference. In addition all children entered in this study were intubated with cuffed endotracheal tubes (Mallinckrodt Medical, Northampton, UK), and a pulmonary arterial line was inserted before the termination of CPB. No child received dexamethasone peri-operatively

Details collected for later analysis as measures of the surgical insult included: minimal operative temperature, duration of cardiopulmonary bypass, duration of aortic cross clamp and circulatory arrest if applicable. Operations were grouped according to technical difficulty utilising a recent classification developed by an 11-member national panel of paediatric cardiologists and cardiac surgeons (Jenkins, Gauvreau and others, 2002).

4.2.3 Clinical care and outcome measures

On return to CICU, patients were mechanically ventilated using volume cycled intermittent positive pressure ventilation (Servo ventilator 900C, Siemens Medical Systems, Solna, Sweden). All patients received continuous infusions of vecuronium (1-3 mcg/kg/hr), morphine (20-40 mcg/kg/hr) and midazolam (1-3 mcg/kg/hr) until stable. The central body temperature was maintained at around 37°C using cooling mattress or warming blanket, and overhead radiant heater. Dopamine, dobutamine, glyceryl trinitrate, and diuretics (usually frusemide) were used as clinically indicated. Volume infusions (usually packed red blood cells or 5% albumin) were given to maintain adequate filling pressures. The patient was extubated upon clinical judgement, and the duration of mechanical ventilation was recorded.

Clinical care was unaffected by participation in this study, and results of investigations were not available to the treating physician. All children received 24 hours of peri-operative antibiotic cover - amikacin and flucloxacillin. If there were clinical concerns about sepsis, additional antibiotics were started at the treating clinician's discretion (amikacin and teicoplanin).

Outcome measures recorded are listed in table 4.1.

Outcome measures
cardiac index
mixed venous saturation
hepatic or renal dysfunction
sepsis
SIRS
length of stay (LOS) in CICU
death

Table 4.1 Outcome measures recorded prospectively.

4.2.3.1 Patient Monitoring

All patients had continuous invasive monitoring of systemic and pulmonary arterial and central venous pressures.

4.2.3.2 Measurement of Cardiac Index (CI) and Mixed Venous Saturation

Arterial and mixed venous blood samples were taken from the peripheral arterial and pulmonary arterial catheters. Sampling was delayed if a change in ventilatory or haemodynamic support had been made in the preceding 15 minutes. Blood samples were analysed for oxygen and carbon dioxide using I-STAT Portable Clinical Analyser (Hewlett-Packard, GmbH Bolingen, Germany Chiron Diagnostics, Halstead, UK). Cardiac output (CO) was then calculated using the direct Fick method, according to the equation:

$$CO = VO_2 / (CaO_2 - CvO_2)$$

where CaO_2 and CvO_2 are arterial and mixed venous oxygen contents respectively. Oxygen consumption (VO_2) was measured with an online respiratory mass spectrometry using mixed expirate inert gas (argon) dilution method (Davies and Denison, 1979; Li, Schulze-Neick and others, 2000). Cardiac Index (CI) was calculated according to body surface area (BSA):

$$CI = CO / BSA$$

Low cardiac index was defined as a cardiac index $< 2 \text{ l/min/m}^2$ at any stage over the 1st 24 hours (Wernovsky, Wypij and others, 1995).

4.2.3.3 Hepatic or Renal Dysfunction

Daily blood was routinely taken on all patients to monitor haematological and biochemical parameters post-operatively. Organ dysfunction was considered to have occurred if one or more of the following criteria were met.

Hepatic dysfunction – total bilirubin $\geq 4 \text{ mg/dL}$

– alanine transaminase ≥ 2 times the upper limit of normal.

Renal dysfunction – creatinine ≥ 2 times upper limit of normal age-adjusted limits

– need for renal replacement therapy

4.2.3.4 Length of Stay

Consistent with the division of length of stay in Chapter 3, long stay was defined as requirement for > 5 days intensive care support

4.2.4 Blood sampling, measurement of MHC Class II expression and cytokines

Whole blood (2ml) was obtained from the arterial line and placed in a sterile container containing 10 units/ml of heparin. Sampling times were as follows: *pre op*, following induction and insertion of arterial line; *X clamp*, on release of the aortic cross clamp; *CPB*, after the end of CPB; *MUF*, following modified ultrafiltration; *CICU*, upon arrival onto the CICU; 2, 4, 8, 14, 18, 24, and 48 hours after admission onto the intensive care unit.

Fifty microlitres of whole blood was removed and incubated with monoclonal antibodies as per the method described previously in chapter 2.3.4 and 3.2.5, to determine monocyte MHC Class II expression. Remaining whole blood was immediately centrifuged at 1500G for 6 minutes, plasma supernatant separated and frozen at -70°C for cytokine analysis.

Stored plasma supernatants were analysed for cytokines using enzyme-linked immunosorbent assays for IL-6, IL-8, TNF- α , IL-10 and IL-1ra as described in Chapter 2.3.5. Each patient's samples were analysed together on a single plate to ensure accuracy of intra-patient comparisons. Optimisation of the assays involved comparison of two commercial cytokine detection systems.

4.2.5 Cytokine load over the first 24 hours

Cytokine levels in the blood vary from minute to minute, have a short half-life, high clearance, and are recognised to have local as well as systemic effects. These characteristics, in combination with the recognised paracrine nature of cytokines, make interpretation of a single plasma measurement at any point in time difficult. In order to ascertain if there was a relationship between cytokine response and the patient's clinical condition, the cytokine load for each patient was calculated. Cytokine load was calculated as the area under the curve from *pre-op* measurement through to 24 hours post admission onto the intensive care unit. This measurement was an approximation, calculated by drawing straight lines between each sequential cytokine measurement. Cytokine load equalled the sum of the area under the curve for each sampling point calculated using the following formula:

$$AUC = C_1 \times (T_2 - T_1) + (T_2 - T_1)(C_2 - C_1)/2$$

AUC = area under curve;

T₂-T₁ = time difference between cytokine measurements (min);

C = cytokine concentration at a particular time point (pg/ml).

4.3 STATISTICS

Log transformations were conducted to normalise the distributions of plasma cytokine levels. Data is expressed as mean \pm SEM unless otherwise stated. Comparisons between data at different time points or between data sets at a single time point were carried out using the Mann-Whitney U test, or Kruskal-Wallis if there were more than 3

data sets. Correlation between two data sets was assessed using Spearman's correlation coefficient. The change of the data over the study period was analysed with a one-way analysis of variance for repeated measures (ANOVA).

4.4 RESULTS

4.4.1 Optimisation of the method for cytokine detection

4.4.1.1 Selection of Capture and Detection antibody.

There is very little literature available on levels of circulating cytokines in well young children. It was expected that young children would have very low levels of cytokines pre-operatively, but that levels may increase several fold following CPB. Published cytokine post-operative cytokine levels in children were: up to 400pg/ml for IL-6, IL-8 (1200pg/ml), TNF- α (60pg/ml), IL-10 (200pg/ml) (Ashraf, Tian and others, 1997;Brix-Christensen, 2001;Butler, Pathi and others, 1996;Seghaye, Grabitz and others, 1996).

In order to ascertain the optimal method for cytokine detection in this patient group, plasma previously collected and stored at -70°C from the patient cohort in Chapter 3 was utilised. Two commercially available cytokine ELISA antibody pairs, both claiming sensitivity in the < 10 pg/ml range, were compared (Fig 4.1). A random selection of 40 pre-operative samples was used. Twenty samples were tested for four different paired anti-cytokine antibodies. Unfortunately there was insufficient sample to test the same sample across all cytokines for both manufacturers.

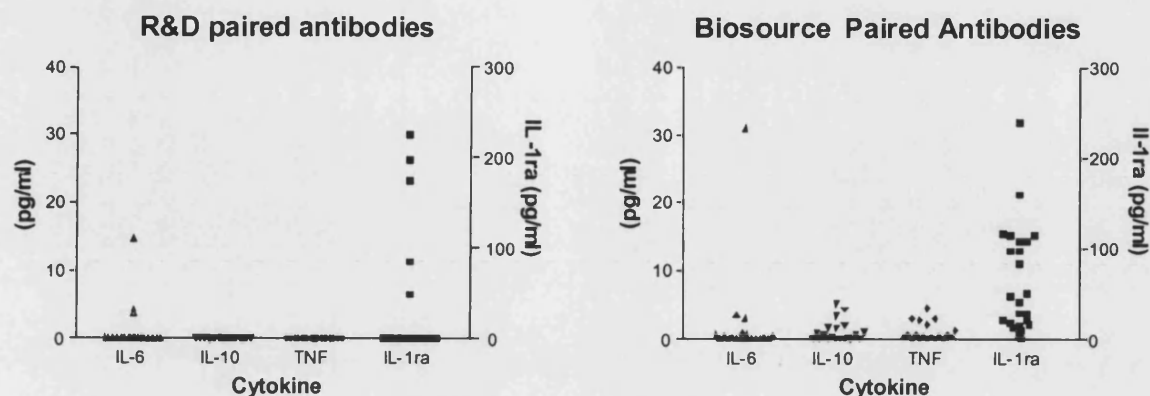


Figure 4.1 Plasma samples from 40 pre-operative cardiac patients were tested against two commercially available cytokine antibody pairs. Allowing for patient variability in resting cytokine levels, the Biosource paired antibodies for the cytokines of interest appeared to be more sensitive at low concentrations than the R&D paired antibodies.

4.4.1.2 Optimisation of manufacturer's recommendations.

A dilutional gradient of capture antibody against detection antibody was performed for each cytokine of interest. The standard curves with a high signal to noise ratio were obtained with a capture antibody at a concentration of $1\mu\text{g/ml}$ for all cytokines except $\text{TNF-}\alpha$ ($5\mu\text{g/ml}$), and a detection antibody concentration of $0.4\mu\text{g/ml}$ except for IL-8 ($0.1\mu\text{g/ml}$) (results not shown).

In order to discriminate differences between samples at the low end of the cytokine range, several steps in the recommended protocol were modified. Best results were obtained by leaving the plasma samples and standards overnight at 4°C before applying the detection antibody rather than the co-application as recommended by the manufacturers. In addition, freshly made up tetramethylbenzidine (TMB) in a phosphate citrate buffer was found to be more reliable than the substrate solution obtained from the manufacturer.

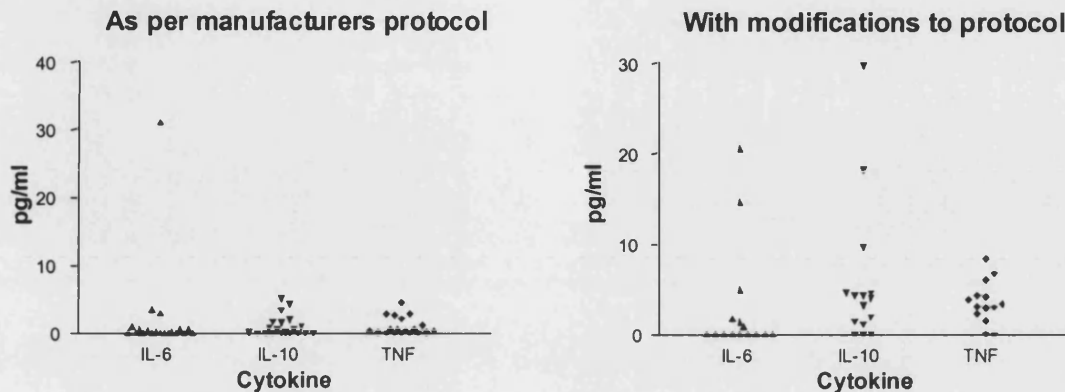


Figure 4.2 Modifications of manufacturers recommendations improved detection of cytokines at lower concentrations. Detection of lower levels of cytokines was improved by leaving the sample and standards in contact with capture antibody at 4°C for at least 12 hours before washing and adding detection antibody.

4.4.1.3 Samples: dilute or neat?

In view of the age of the children and the number of blood samples required, every effort was made to minimize blood volume required at each sample point. The Biosource paired antibodies recommended 100µl of sample for each ELISA well. Utilising the plasma stored from Chapter 3, serial dilutions of samples taken over the peri-operative period were tested. It was apparent that neat samples were required in order to detect subtle changes in IL-6, IL-8 and IL-10. IL-1ra in contrast, could be adequately assayed at a 1 in 10 dilution. Attempts at reducing the volume of sample placed in each well to 50µl gave unreliable results with increased coefficient of variation between replicas (results not shown).

4.4.2 Patient Characteristics and Operative Details

Forty-six infants and children aged between 9 days and 24 months were entered into the study. Thirty-nine patients completed the study protocol, five of whom were neonates. The seven patients who did not complete the study protocol were withdrawn at the request of the operating surgeon during the surgical procedure. There was no difference between the patients studied and those who failed to complete the study in terms of patient characteristics (Table 4.2). Longer bypass time, cross-clamp time, and greater proportion requiring circulatory arrest, suggested that the group that did not complete had a more complicated intra-operative course. Two patients refused to participate.

	Study Group	Drop-out Group
Number	39	7
Age	6.5mths (9days-24mths)	5mths (12days-19mths)
Sex (m:f)	22:17	3:4
Weight	6.35kg (2.5-12.9kg)	5.1kg (3.3-10kg)
Operation: VSD	41%	43%
AVSD	17%	14%
Switch	11%	14%
TOF	11%	14%
other	20%	14%
CPB duration	107min (49-187min)	172min (87-432)
Aortic X-clamp duration	56min (19-125 min)	69min (41-93)
Circulatory arrest time	n=8 24min (2-32min)	n=3 21min (15-24min)

Table 4.2: Patient characteristics and operative details.

39 patients completed the study protocol, 7 patients failed to complete the protocol and are not included in the analysis. The patients which failed to complete the study were similar to the study group in demographics but had longer operations with a greater requirement for circulatory arrest, suggesting a more technically difficult operative repair. X-clamp =aortic cross clamp.

4.4.3 Outcome

Consistent with the study described in Chapter 3, length of stay was bimodally distributed, with 28 of the 39 patients (71%) staying less than 5 days, and 11 of the 39 (28%) requiring more than 5 days intensive care (median stay 17 days; 6 – 86 days). Two patients died during the study, one in the short stay group and one in the long stay group. The first patient died on the day of surgery from an intracerebral bleed that occurred intraoperatively. The second patient died 32 days post surgery from septic complications. Both data sets are included in the analysis. Three patients fulfilled the criteria for sepsis, and an additional 1 patient for SIRS. One other patient had his wound re-explored for local infection but did not fulfil the criteria for either sepsis or SIRS.

Two patients required renal support during the post operative period, one of whom was septic. There were no patients with significant hepatic dysfunction. Whilst the percentage of patients with proven sepsis in this group was lower than in Chapter 3 (3/39, 7.6% vs 11/82, 11%), 23 of the 39 patients had a complicated post-operative course (59% vs 46% of patients in Chapter 3). These complications included 3 patients needing to return to theatre, whilst a further 3 patients had their chest reopened or delayed sternal closure. Five patients had poor cardiac function, one of whom required extracorporeal membrane oxygenation (ECMO) support.

There was an overall impression that in addition to being younger (median age 6.5 vs 10 months), this study group had a more complicated early post-operative period than patients studied in Chapter 3. However Figure 4.3 shows that there was no significant difference in the physiological state of the study groups on arrival into CICU using the risk of mortality (PIM) score ($p=0.78$).

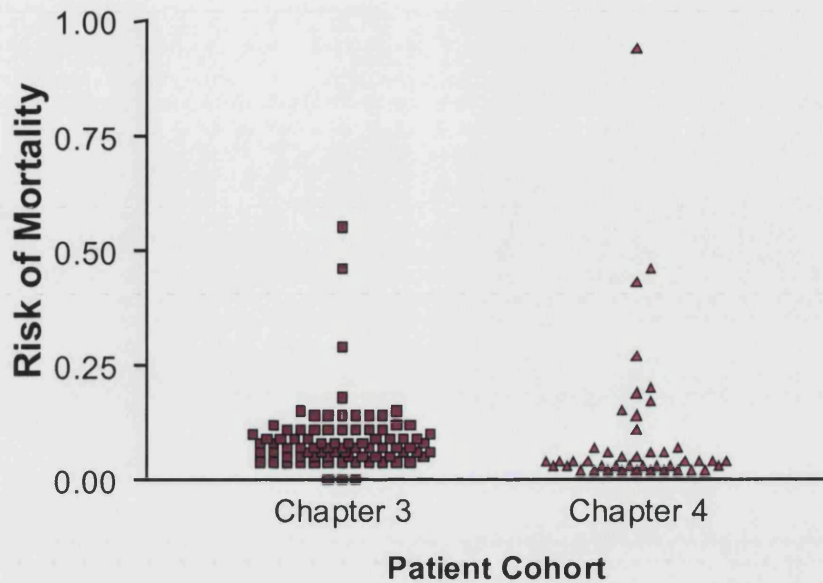


Figure 4.3 The risk of mortality score is plotted for each study patient in the Chapter 3 and 4 cohorts.

4.4.4 Cytokine profile in paediatric patients undergoing CPB

A primary aim of this study was to describe in detail the cytokine profiles of both pro- and anti-inflammatory cytokines pre-operatively and over the first 48 hours post surgery (Fig 4.4). Levels of all cytokines studied approached the lower limit of detection pre-operatively with the exception of IL-1ra. Plasma cytokine levels rose following the initiation of cardiac surgery and CPB. Levels of IL-10 peaked at the end of surgery following modified ultrafiltration, whilst IL-6, -8, and TNF- α peaked soon after on arrival to CICU (paired t-test $p < 0.001$ for all except TNF- α). IL-1ra progressively rose until 4 hours post-operatively. Circulating levels of IL-6, -8, -10, and IL-1ra remained significantly elevated from the pre-operative levels at 8 and 24 hours post op ($p < 0.001$).

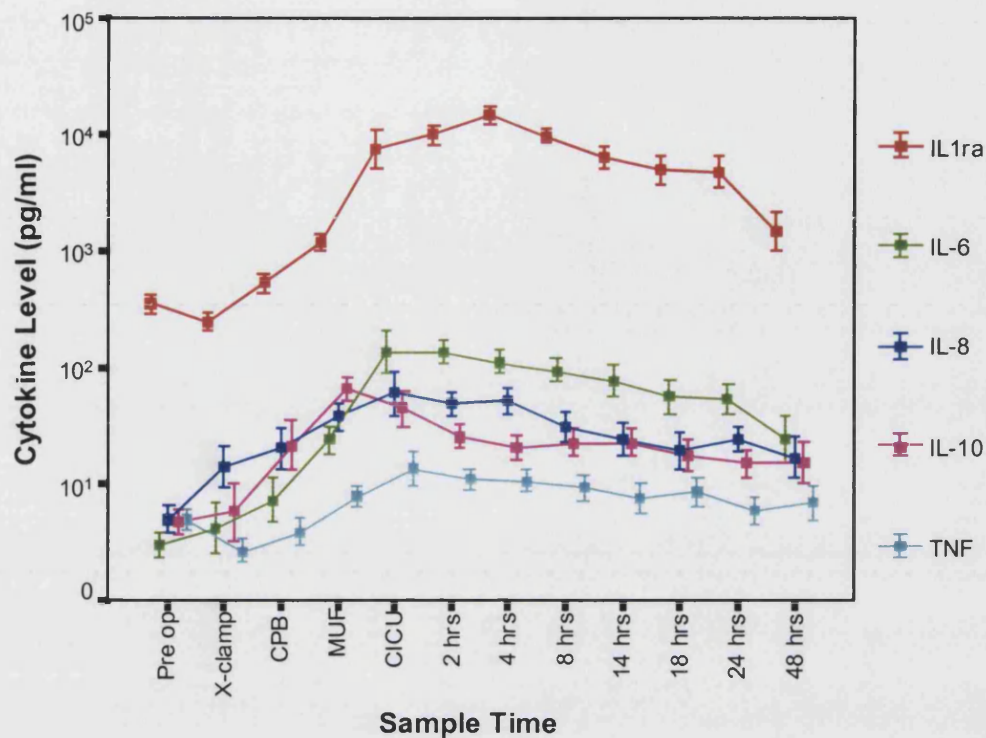


Figure 4.4 Systemic pro-inflammatory and anti-inflammatory cytokine responses to cardiopulmonary bypass in paediatric patients. Plasma samples were collected at induction of anaesthesia (pre op), at the end of surgery following modified ultrafiltration (MUF), on arrival onto CICU (CICU), and at set intervals over the first 48 hours. Plasma was spun immediately and supernatant collected for analysis of cytokine levels by ELISA. Results are presented as mean of the logged cytokine level \pm SEM. All cytokines, except TNF- α , were significantly elevated on arrival to CICU and remained significantly elevated at 24 hours.

The greatest increase was seen in IL-6 (mean increase 820x baseline; 95%ci 0-1733), the least increase in TNF- α (48x baseline; 95%ci 0-122).

4.4.5 The systemic cytokine response to surgical insult

As discussed in the introduction (Section 1.2.2), it is assumed that increases in circulating cytokines following cardiac surgery are the result of pro-inflammatory triggers that occur intra-operatively. To investigate this assumption further, both peak

cytokine levels and cytokine load in this patient population, were examined against measures of surgical insult, including cardiopulmonary bypass time, aortic cross-clamp time, circulatory arrest time, and lowest temperature at which the operation was performed. IL-6 and IL-8 levels 90 minutes after releasing cross-clamp have been shown in adult studies to significantly correlate with ischemic time (Wan, Marchant and others, 1996). In this patient group, there was only a weak positive correlation between peak IL-6 and IL-8 levels in the postoperative period and CPB time (Spearman correlation coefficient $r=0.537$ and $r=0.596$ respectively, $p \leq 0.01$), and IL-6, IL-8 and IL-10 levels and ischemic time ($r=0.460$, $p < 0.01$; $r=0.445$, $p < 0.01$; $r=0.343$ $p < 0.05$ respectively) (Fig 4.5). No correlation was found between cytokine load over the first 24 hours and any of the measures of surgical insult.

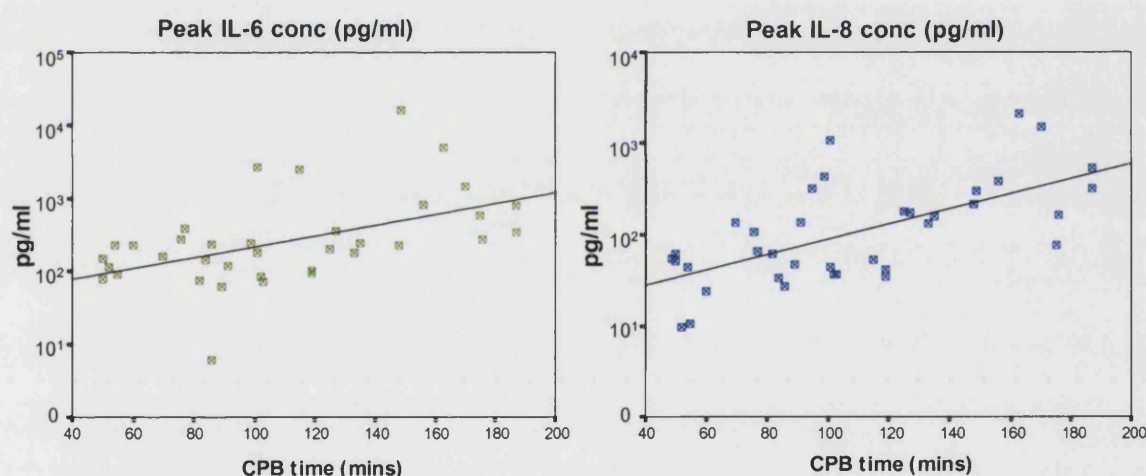


Figure 4.5 Correlation between duration of CPB and peak concentration of IL-6 and IL-8 post-operatively. Only a weak positive correlation was found between the duration of CPB and peak systemic levels of IL-6 and IL-8 ($r=0.537$ and 0.596 respectively).

Active cooling during the cardiac repair was designed in an effort to reduce cellular metabolic rate and preserve end organ function. In this study, there was a suggestion that the greatest peaks in all cytokines were seen in the **more** actively cooled patients.

Dividing the study group into those actively cooled to 18°C or less (operations involving circulatory arrest) and those cooled to between 19-27°C and those maintained at $\geq 28^\circ\text{C}$, showed there was a trend for higher peak cytokine levels in the more actively cooled patients (Kruskal-Wallis $p=0.06$ for all cytokines except IL-1ra) (Fig 4.6). Significantly higher peak concentrations of IL-6 were present in the circulatory arrest group (Mann-Whitney U $p<0.03$) compared to those operated at $\geq 28^\circ\text{C}$. Whilst peak concentrations appeared affected by temperature, overall cytokine load in the first 24 hours was not (results not shown). This suggests that the same cytokine response may be occurring but that low temperatures result in a more rapid and higher initial response.

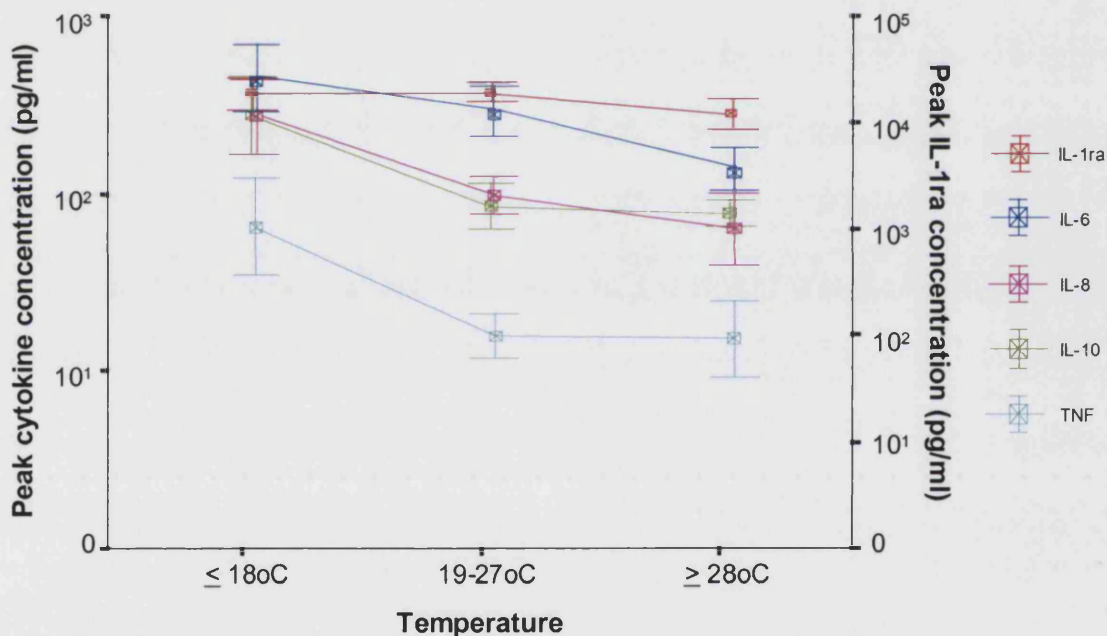


Figure 4.6 A trend toward higher peak cytokine levels were seen in those patients operated on at a lower temperature.

Minimum operative temperature was grouped as extreme hypothermia ($\leq 18^\circ\text{C}$), moderate hypothermia (19-27°C), and mild hypothermia ($\geq 28^\circ\text{C}$). There was a trend toward peak cytokine levels increasing as operating temperatures decreased ($p=0.06$) (exception IL-1ra). This reached significance for IL-6 when levels at $\leq 18^\circ\text{C}$ were compared to levels produced at $> 28^\circ\text{C}$ ($p<0.03$).

Recently it has been suggested that blood cardioplegia reduces the inflammatory response to cardiopulmonary bypass (Billia, Carter and others, 2002; Liebold, Langhammer and others, 1999; Wan, Yim and others, 1999). In this study 46% (18/39) of patients received blood cardioplegia. Those patients with blood cardioplegia did not differ in age, gender, CPB time, X-clamp time, or lowest operative temperature, from patients managed with crystalloid cardioplegia. Nor was there any significant difference in cytokine response (peak or load) between patients managed with blood vs. crystalloid cardioplegia.

4.4.6 The timing of the decline in cardiac function varied in relation to release of the aortic cross clamp

The decline in cardiac function over the first post-operative night was measured using Cardiac Index (CI) in 36 of the 39 patients. Twenty-one (58%) of these patients had a CI measurement of < 2 l/min/m² at some time in the 1st 24 post operative hours (Fig 4.7a). The timing of the lowest recorded cardiac index was very variable. Eight patients (22%) had their lowest reading at 2 hours post arrival onto CICU, while 6 patients (17%) had their lowest reading at a time point corresponding to Wernovsky's findings (8 hours). Overall, 75% of patients studied had their lowest cardiac index within the first 8 hours of arriving onto CICU. Accepting that this was a heterogeneous group of patients, there was no pattern to suggest a decline in cardiac function at a set time following reperfusion.

There was no correlation between the lowest cardiac index measured during the first 24 post-operative hours and CPB time, aortic cross-clamp time, or lowest operating temperature (4.7b). Nor was there a difference in range of cardiac indices between patients operated with and without circulatory arrest.

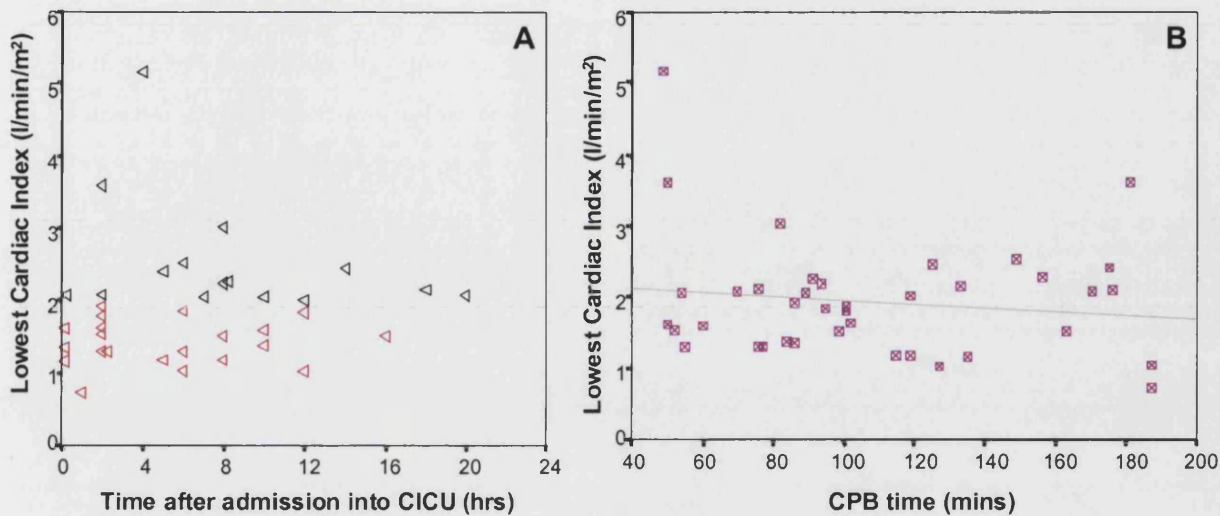


Figure 4.7 Scatter plots of timing of lowest cardiac index and relationship to duration of CPB.

Thirty-six of the study patients had cardiac index measurements recorded over the first 24 hours post-operatively. 75% of patients had their lowest measurement within the first 8 hours, however there was no clear nadir in cardiac function at the 8 hour time point (A). The lowest cardiac index in the first post-operative night appeared to be unrelated to the duration of CPB (B).

4.4.7 Poor cardiac function during the first post-operative night is associated with higher circulating levels of TNF- α

Those patients who had poor cardiac function (defined as cardiac index < 2 l/min/m²) over the first 24 hours post-operatively had higher levels of circulating TNF- α when compared to those patients whose CI remained ≥ 2 l/min/m² (ANOVA $p < 0.05$) (Fig 4.8).

Whilst the literature in adults suggests that both IL-6 and IL-8 are myocardial depressants, the cytokine profiles for these cytokines were not significantly higher in patients whose cardiac index fell to < 2 l/min/m² over the first post-operative night (Finkel, Oddis and others, 1992; Hennein, Ebba and others, 1994; Kawamura, Inada and others, 1999; Kawamura, Wakusawa and others, 1993) (Fig 4.8 & 4.9).

No relationship was found between the timing of peak pro-inflammatory cytokine concentration and timing of lowest recorded cardiac index.

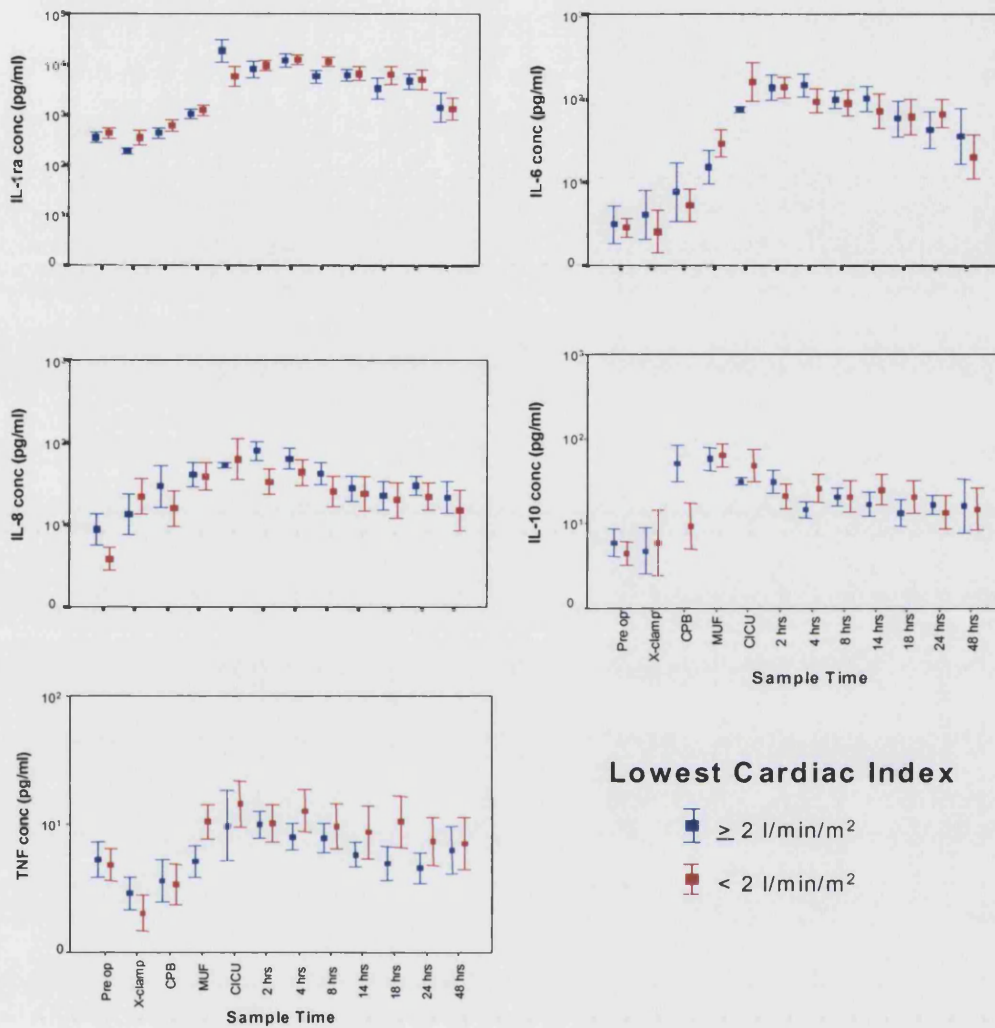


Figure 4.8 Cytokine profiles divided according to post-operative cardiac function. Patients whose CI fell to < 2 l/min/m² had significantly higher levels of circulating TNF- α over the first post-operative night (ANOVA $p < 0.05$). There was no significant difference in the IL-6 or the IL-8 cytokine profiles in patients divided by cardiac function.

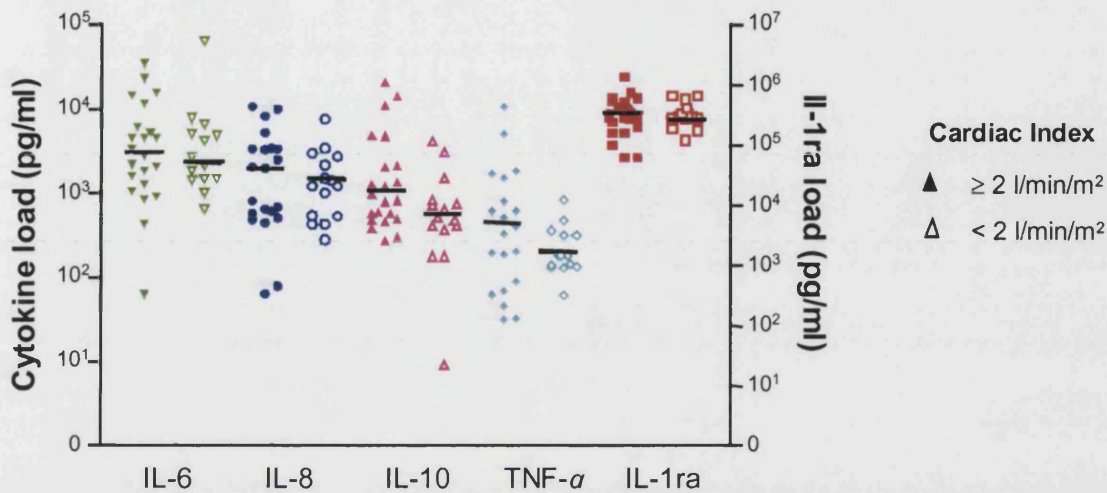


Figure 4.9 Patients were divided according to their lowest measured cardiac index in the first post-operative 24 hours and cytokine load during this period examined. Those patients with poor cardiac function in the early postoperative period, tended to have a greater systemic load of TNF- α and IL-10 in the first 24 hours ($p=0.07$ and 0.08 respectively). Solid shape; CI ≥ 2 l/min/m², open shape; CI < 2 l/min/m².

4.4.8 The systemic cytokine response and outcome

Whilst cytokine levels were only weakly correlated with measures of surgical insult (Section 4.4.5), cytokine levels in the post-operative period were significantly higher in patients who subsequently required prolonged support (ANOVA $p<0.05$)(Fig 4.10).

The frequency of other outcome measures, including organ dysfunction, sepsis/SIRS, and death was too low to allow for meaningful analysis.

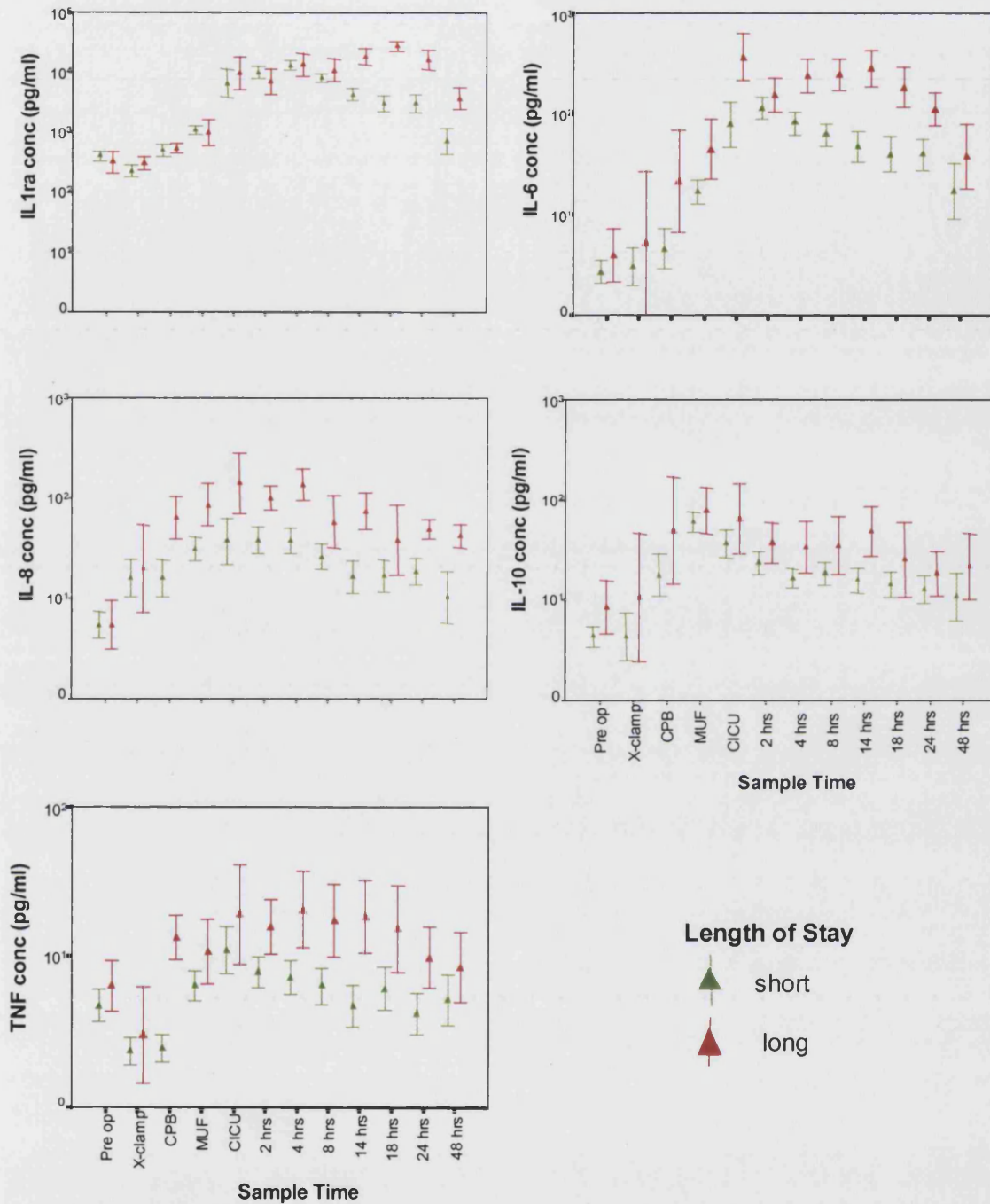


Figure 4.10 Significantly higher cytokine levels over the first 24 hours post-operatively were seen in those patients that subsequently required prolonged support. One notable difference was that circulating levels of the pro-inflammatory cytokines persisted at levels similar to those generated at the end of surgery over the early post-operative period in long stay patients, whilst levels in short stay patients declined. This pattern also occurred for IL-1ra.

4.4.9 Monocyte MHC Class II expression and response to surgical insult

Monocyte MHC Class II surface expression was measured over the first 48 hours post operatively. The median MHC Class II expression pre-operatively was 94% (S.D \pm 17). Cardiac surgery resulted in significantly reduction in surface expression of MHC Class II in all patients compared to preoperative levels ($p < 0.001$). The reduction in expression postoperatively was greater in this study compared to the results presented in Chapter 3. There was no correlation between the reduction in MHC Class II expression and age, sex, measures of surgical insult (surgical group, lowest temperature, cardiopulmonary bypass time, cross-clamp time, circulatory arrest time) or physiological state on arrival onto CICU (PIM score, Risk of Mortality score). Figure 4.11 shows that there was no relationship between the level of reduction of MHC Class II expression post-operatively and the degree of cooling the patient underwent during surgery.

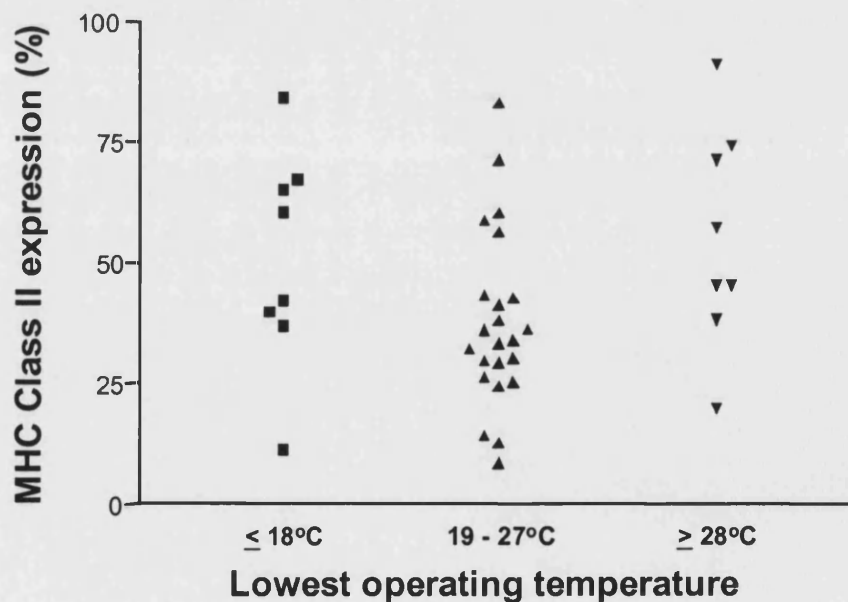


Figure 4.11 Lowest monocyte MHC Class II expression and operative temperature. The lowest temperature at which the patient was operated did not appear to affect the degree of reduction in monocyte HLA-DR expression seen post-op.

4.4.10 Monocyte MHC Class II expression and cytokine response to surgery

A weak negative correlation was found between systemic anti-inflammatory cytokine levels and monocyte surface MHC Class II expression (Spearman correlation coefficient $r=-0.222$ and $r=-0.277$, $p\leq 0.001$ for IL-10 and IL-1ra respectively) (Fig 4.12). There was no relationship between systemic levels of TNF- α (peak or load) and MHC Class II expression on circulating monocytes.

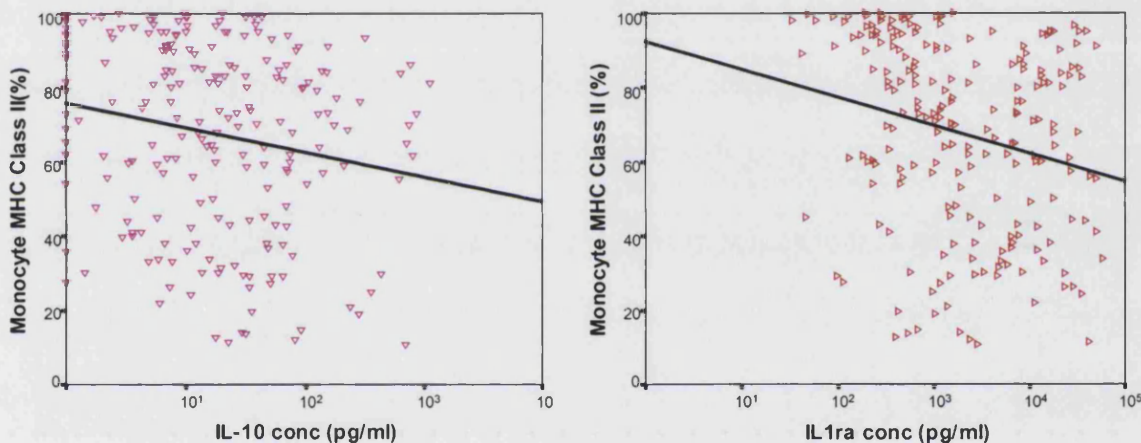


Figure 4.12 Correlation between high levels of systemic anti-inflammatory cytokines and low MHC Class II expression on circulating monocytes. There was a weak negative correlation between IL-10 or IL-1ra and levels of monocyte MHC Class II expression ($r=-0.222$ and $r=-0.277$, $p\leq 0.001$). This correlation was not present when lowest MHC Class II expression was examined against peak IL-10 or peak IL-1ra.

Whilst impossible to test with such a small study population, the fall in MHC Class II on monocytes during theatre appeared to coincide with the rise in circulating IL-10 levels, with a slight recovery post-operatively that mirrored the increase in TNF- α levels (Fig 4.13).

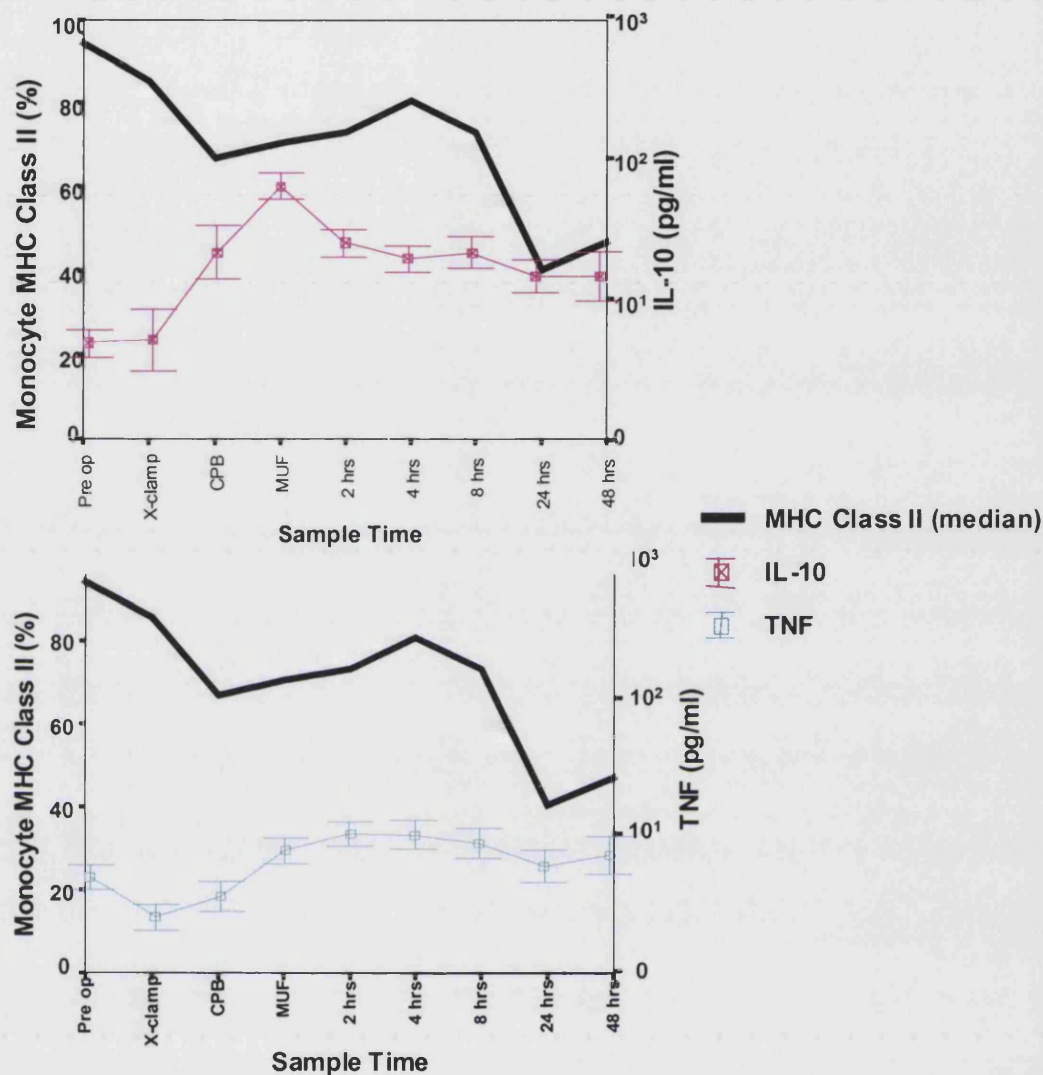


Figure 4.13 IL-10, TNF- α and monocyte surface MHC Class II expression. The overall change in monocyte MHC Class II expression (%) for the patient group is plotted against the systemic levels of IL-10 and TNF- α (mean & SEM). The rise in systemic IL-10 levels during theatre corresponded in time to a fall in MHC Class II expression, whilst the direction of the TNF- α curve appears to follow the MHC Class II curve.

Whilst there was no difference in overall pro-inflammatory cytokine exposure (cytokine load), those patient's whose MHC Class II expression fell to $< 60\%$ in the 1st 72 hours had significantly later peaks in pro-inflammatory cytokines (IL-6, IL-8, and TNF- α), compared to those patients who maintained their monocyte MHC Class II expression $\geq 60\%$ (Mann-Whitney $p < 0.05$) (Fig 4.14).

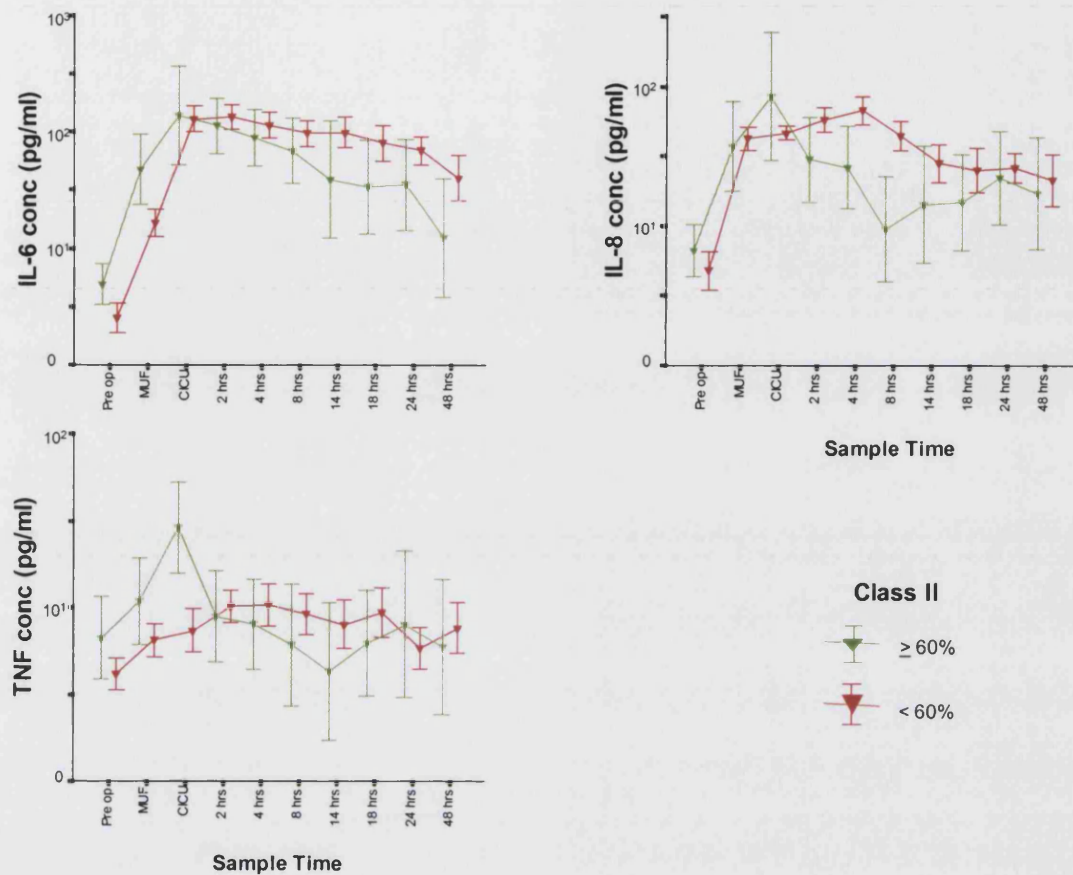


Figure 4.14 Patient's systemic pro-inflammatory cytokine response divided according to the lowest MHC Class II expression over the same time period. Patient's whose monocyte Class II expression fell to $< 60\%$ in the first 72 hours had significantly later pro-inflammatory cytokine peaks (Mann-Whitney $p < 0.05$). There was no difference in the timing of the anti-inflammatory response. Overall pro-inflammatory cytokine exposure (cytokine load) did not differ significantly between the two groups.

4.4.11 Monocyte MHC Class II expression and outcome

There did not appear to be any direct relationship between low monocyte MHC Class II expression and cardiac function postoperatively (Fig 4.15). There was no difference in surface expression of MHC Class II at 24 or 48 hours between those children with low

cardiac index ($< 2 \text{ l/min/m}^2$) and children with cardiac index $\geq 2 \text{ l/min/m}^2$, suggesting that low cardiac function does not appear to predispose the patient to reduced MHC Class II expression on the surface of circulation monocytes.

Three children developed sepsis and one child developed SIRS in the post operative period. Two children died during the study (1 septic, 1 from non-septic causes). All these children had a reduction in MHC Class II expression to $< 60\%$ post op.

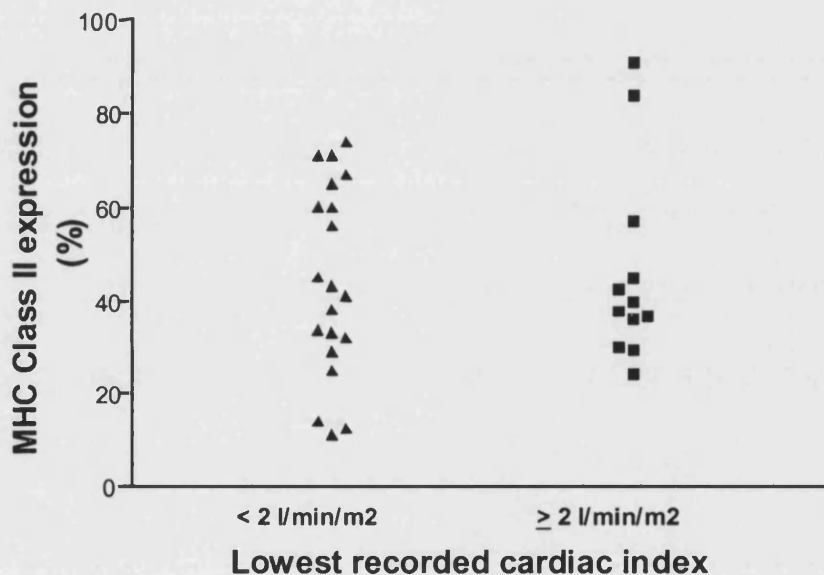


Figure 4.15 Lowest monocyte MHC Class II expression was examined against normal ($\geq 2 \text{ l/min/m}^2$) or poor cardiac function ($< 2 \text{ l/min/m}^2$). Patients with poor cardiac function in the post operative period did not have significantly lower monocyte MHC Class II surface expression.

Patients requiring greater than 5 days in Intensive Care had a trend toward greater reduction in monocyte MHC Class II expression at 24 and 48 hours compared to those who were discharged within 5 days (Fig 4.16). This difference did not reach significance in the small number studied. All but 1 of the long stay patients had a fall in MHC Class II expression to $< 60\%$ in the first 48 hours postoperatively.

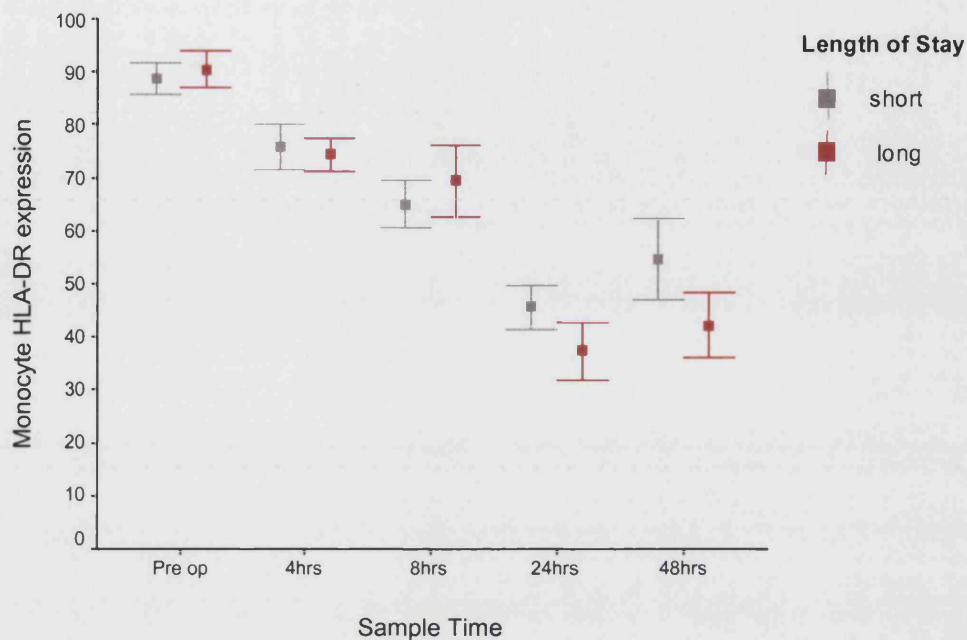


Figure 4.16 The reduction in surface expression of MHC Class II on monocytes was greatest in those patients who required prolonged Intensive Care support.

Monocyte surface expression of MHC Class II fell in all patients following cardiopulmonary bypass. At 24 and 48 hours this fall was greater in those patients that subsequently required > 5 days intensive care support. (mean ± SEM)

4.4.12 Cytokine Balance

Increasingly, interest has centred on an attempt to quantify the balance of the inflammatory response to a critical illness or insult. To date there is no accepted method to quantitate cytokine balance. Consistent with published data on cytokine interactions/feedback, there were a number of positive correlations between pro- and anti-inflammatory cytokines in this study. Consistent with the effect TNF- α is known to have on IL-10, there was a positive correlation between IL-10 load and the TNF- α load over the first 24 hours ($r=0.622$, $p<0.001$) (Fig 4.17). A positive correlation was also seen between both the peak and load IL-10 levels and peak and load IL-8 levels ($r=0.567$, $p<0.001$ and $r=0.643$, $p<0.001$ respectively).

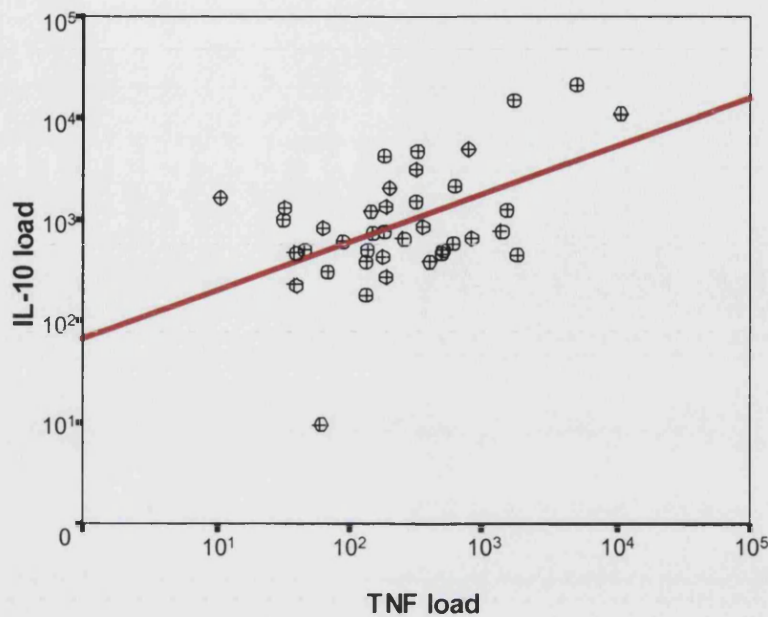


Figure 4.17 There was a positive correlation between the IL-10 and the TNF- α load over the first 24 post-operative hours ($r=0.622$, $p<0.001$).

Previously published attempts to quantitate cytokine balance include IL-10 to TNF- α ratio and IL-10 to IL-6 ratio (Hovels-Gurich, Schumacher and others, 2002). If cytokine imbalance is a major factor in the development of low MHC Class II expression, poor cardiac index, and need for prolonged support, these previously published cytokine ratios were not reflective of this imbalance in this patient population (Fig 4.18).

4.4.13 Age and gender

In contrast to other paediatric studies (Alcaraz, Sancho and others, 2002; Trotter, Muck and others, 2001), this study showed no difference in cytokine levels or post operative morbidity between neonates & non-newborns, or males & females.

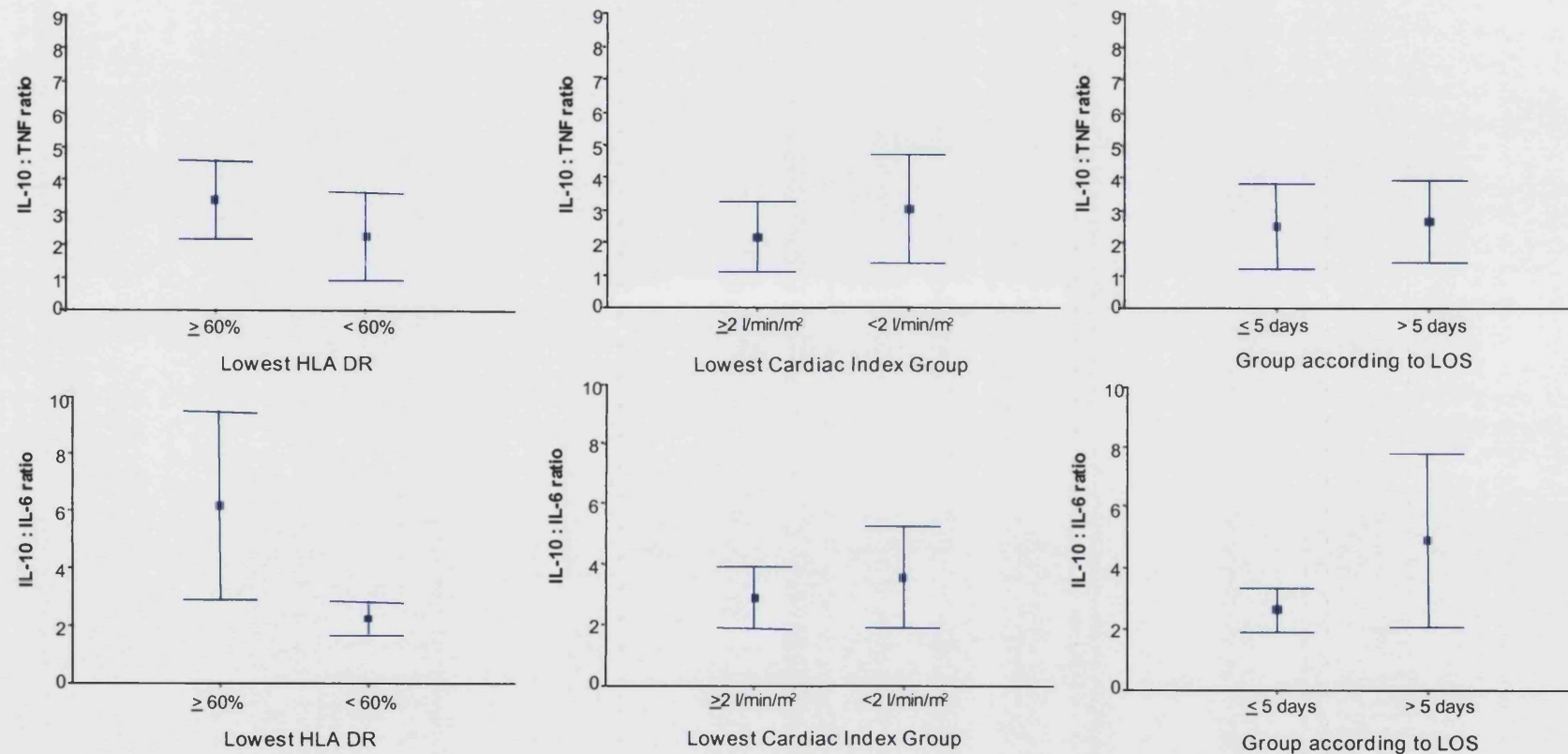


Figure 4.18 Lowest monocyte MHC Class II expression, lowest cardiac index, and group according to length of stay were examined against IL-10:TNF- α ratio and IL-10:IL-6 ratio. No significant differences were seen between previously published measures of cytokine balance and the three parameters shown.

4.5 DISCUSSION

This study describes in detail the early cytokine response to cardiac surgery involving CPB. It shows that both pro- and anti-inflammatory cytokines rise simultaneously from as early as release of aortic cross-clamp and that peak cytokine concentrations occur within the first 2 hours (4hrs for IL-1ra) of the child arriving onto the intensive care unit. Both pro- and anti-inflammatory cytokines remain significantly elevated at 24 hours post-op.

4.5.1 Effects of Temperature

The results presented here suggest that active cooling to $\leq 18^{\circ}\text{C}$ increases the inflammatory response to cardiac surgery. Since late 1960 / early 1970, congenital heart defects in newborns and infants have been corrected by using deep hypothermic circulatory arrest. This was thought to both protect the heart and minimise inflammation. However, with the development of concerns about neurological morbidity associated with prolonged periods of circulatory arrest, moderate hypothermia and low flow cardiopulmonary bypass has gained favour. Whilst many congenital heart defects can be operated on with a continuous perfusion technique, circulatory arrest provides excellent surgical exposure in a small heart by eliminating the need for multiple canulas within the field. Thus in some anomalies it is still the preferable technique. In previous paediatric studies, deep hypothermia ($\leq 18^{\circ}\text{C}$) and circulatory arrest has been associated with lower levels of pro-inflammatory cytokines (Tassani, Barankay and others, 2002). However animal models have suggested that the effect of temperature on inflammatory response is not linear. A study in pigs suggested that moderate hypothermia (28°C) had lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines compared to either normothermia (37°C) or deep hypothermia ($\leq 20^{\circ}\text{C}$) (Qing, Vazquez-Jimenez and others, 2001; Wan, DeSmet and others, 1996). This and other studies have also suggested that moderate hypothermia is associated with a lower histological organ damage score (Vazquez-Jimenez, Qing and others, 2001).

The results presented here showed a trend toward increasing cytokine levels at lower operative temperatures. Whilst more complex surgery is often performed at 18°C under circulatory arrest, this trend is unlikely to be due to complexity of surgery alone as there was poor correlation between duration of circulatory arrest and cytokine response and only a weak correlation with duration of aortic cross-clamp. The high cytokine response seen in patients operated on at $\leq 18^\circ\text{C}$ may be a direct effect of temperature. Temperatures $< 20^\circ\text{C}$ have been shown to disrupt general cellular function and cause cell membrane damage through disruption of membrane lipids (Cohen, Ophir and others, 1990; Funk, Wunderlich, and Kreutz, 1982; Gousset, Wolkers and others, 2002; Ramlov, 2000; Tablin, Wolkers and others, 2001). Whilst moderate hypothermia may decrease inflammation, extreme hypothermia may result in general cell membrane disruption and exacerbate the inflammatory response seen following bypass.

4.5.2 Cardiac Function

There was only a weak correlation between peak levels of pro-inflammatory cytokines (IL-6 and IL-8) and duration of cardiopulmonary bypass and ischaemia-reperfusion. However, as discussed earlier in section 4.1, these may not be good surrogates for surgical insult. In this study it was possible to measure cardiac function using cardiac index. This is both time consuming and invasive and is rarely performed in children. From the 39 patients studied, there was no clear pattern of a decline in cardiac function during the 12 hours following surgery. This contrasts with the data of Wernovsky who studied 171 neonates and infants all following an arterial switch procedure. They found a clear nadir in cardiac function 9 and 11 hours post reperfusion (Wernovsky, Wypij and others, 1995). While the study groups differ in heterogeneity of the underlying cardiac lesion, it was surprising that the study presented here failed to show even a trend towards this post-operative nadir.

Despite the assumption that TNF- α plays an important role in '*post-pump syndrome*' only very low levels of TNF- α were found in this population. However, the only significant finding with regards to cytokines and cardiac function was an increase in TNF- α levels post-operatively in those children whose cardiac index fell to $< 2 \text{ l/min/m}^2$. Systemic levels of the other known myocardial depressants (IL-6 and IL-8) were not particularly elevated in those children with poor post-operative cardiac

function. This difference in TNF- α is particularly interesting when considering that plasma cytokine levels may not reflect cytokine levels produced locally within tissues. This is important as levels of both TNF- α and IL-6 in patients undergoing CPB have been shown to be significantly higher in coronary sinus blood than in arterial blood, indicating that the myocardium is a major source of these pro-inflammatory cytokines (Wan, DeSmet and others, 1996). The small difference in TNF- α seen systemically in those patients with poor cardiac function may therefore represent a much greater difference in TNF- α produced by the myocardium. To investigate this further, it would require a study such as that by performed by Wan et al (1996) involving sampling of blood from the coronary sinuses. This is unlikely to get ethical approval in a paediatric patient base (Wan, DeSmet and others, 1996).

The only other finding of note was that IL-10 levels at the end of bypass appeared to be greater in those patients who subsequently had good cardiac function. Interestingly, until this time point, circulating levels of TNF- α between the two groups were similar. After the IL-10 rise however, TNF- α levels fell in those patients who maintained good cardiac function. The numbers studied were insufficient for this difference to be significant, but it raises an interesting hypothesis. Could the degree of anti-inflammatory response elicited by surgery (level at end of CPB) determine the degree of inflammation present over the first post-operative night? This hypothesis will be tested in a subsequent study.

4.5.3 MHC Class II expression

Monocyte MHC Class II expression following bypass was significantly reduced. In keeping with chapter 3, the degree of reduction of MHC Class II expression was unrelated to surgical insult, or measures of post-operative cardiac function, but greatest in those children who subsequently required prolonged intensive care support. MHC Class II expression fell to < 60 % in all children who developed sepsis/SIRS or died during this study.

In this patient group, a direct relationship between circulating cytokine levels and reduction in MHC Class II expression on monocytes did not exist. Nor was an altered

cytokine balance (as measured by IL-10:TNF- α and IL-10:IL-6 ratios) present in those patients who had the greatest fall in MHC Class II. So the aetiology of the reduction in MHC Class II expression in the post-operative period remains unclear.

The number of children whose MHC Class II expression fell post-operatively to < 60% was greater in this study population compared to the population studied in Chapter 3 (29/39, 74% compared to 21/82, 26%). The reason for this is unclear. This study involved smaller patient numbers and was restricted to younger children (9 days – 24 months, median age 6.5 months vs 2 days – 16 years, median age 10 months). Age however, is unlikely to be the explanation as the reduction in MHC Class II expression in the previous study was not skewed towards children ≤ 2 years (24% of children ≤ 2 years had reduction in MHC Class II expression to < 60% vs 27% of those children > 2 years). By selecting out just the children ≤ 2 years in both chapter 3 and 4, it can be clearly shown, that the reduction in MHC Class II expression was significantly greater in the children described in chapter 4 (Table below).

	Mean Class II expression % (\pm S.D)	
Timing of Samples	Chapter 3 Study	Chapter 4 Study
Pre op	93% \pm 16%	87% \pm 18%
Day 1	78% \pm 21%	42% \pm 18
Day 2	78% \pm 23%	49% \pm 22%
Day 3	80% \pm 21%	

As previously noted in section 4.4.2, the small patient group presented in this chapter appeared to have more immediate postoperative complications unrelated to sepsis. This may be in keeping with the observation of lower MHC Class II expression. The question then arises, ‘Why did younger age, lower MHC Class II expression and increased frequency of early post-operative complications not translate into a greater proportion of patients requiring prolonged intensive care support?’

Technical problems with MHC Class II staining are a possibility. Throughout both Chapter 3 and 4, the flow cytometer was calibrated regularly, and all samples were

handled according to the optimal staining and analysing method previously determined (section 3.4.2 & 3.4.3). All samples were handled by a single operator. During the course of this study and the study presented in chapter 3, the monoclonal antibodies used for staining were changed several times. With each change, samples at the time were co-stained using both the old and new antibody to ensure comparable results. Several samples were also randomly checked with the specific HLA-DR specific monoclonal antibody. In addition the consistently high pre-operative MHC Class II expression (%) in patients that subsequently had a marked reduction in expression post-operatively, argues against the aetiology being technical.

This study design necessitated a study clinician to be present at the bedside for the first 24 hours. Whilst investigation results were not available to the clinical team, the presence of the study clinician may have had minor affects on patient management. Fluid boluses may have been given earlier and more frequently than normal, nursing staff may have been more attentive to procedures. Such influence may have resulted in earlier stabilisation, earlier detection and treatment of any complications, and potential for earlier extubation and discharge from the intensive care unit. Equally the presence of the study clinician could have placed the patient at increased risk of nosocomial infection because of additional invasive equipment and additional sampling from central lines. These results however suggest a low rather than high incidence of sepsis (3/39, 8% compared with 11/85, 13%, chapter 3).

It has been shown that some medication can itself influence MHC Class II expression. One such drug is fentanyl. Anaesthetic doses of fentanyl have been shown to downregulate surface expression of HLA-DR molecules on circulating monocytes *in vivo* (Carrera, Catala and others, 1993; McBride, Armstrong and others, 1995; Prieto, Subira and others, 1989). Fentanyl was used to a similar extent in both patient groups. However one change that has occurred is a shift from dobutamine to dopamine as a first line inotrope in the immediate post-operative period. This has resulted in part from the personal preference of the different operating surgeons, and in part because of the increased use of milrinone as a vasodilator. There is some evidence to suggest that dopamine acts via increased prolactin to decrease surface expression of MHC Class II on monocytes (personal communication Dr J.Carcillo, USA, 2003). The tantalising

prospect that all patients are experiencing a greater reduction in their monocyte MHC Class II expression because of what would be clinically considered a trivial drug difference begs further investigation, however not during this thesis.

The finding of low MHC Class II expression (<60%) without the association of requirement for prolonged support, or increased incidence of sepsis highlights that reduced MHC Class II expression alone is not sufficient to condemn a patient to a complicated recovery. However, the presence of reduced monocyte MHC Class II expression may highlight a group of patients that are immunoparalysed and unable to respond to a second post-operative insult if discharge from the intensive care unit is not imminent.

4.5.4 Summary

Whilst this is a relatively small study and limited to children two years and under, it suggests that there are significant differences in the cytokine response of children compared to adults following cardiopulmonary bypass. Pro- and anti-inflammatory cytokines rose simultaneously in the early post-operative period and were only weakly related to surgical insult. In addition, systemic levels of TNF- α appear to be greater in those children who have a decline in cardiac function during the first post-operative night, raising the possibility that the biological effects of TNF- α are being underestimated by the low or absent measurements of this cytokine systemically.

Finally, the postoperative reduction in monocyte Class II expression appears unrelated to the cytokine response to surgery, or to measures of cardiac function, but was greatest in those children who subsequently required prolonged support.

CHAPTER 5

Immune Paresis following Cardiopulmonary Bypass

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5.1 INTRODUCTION

The results presented in chapter 4 demonstrated that cardiac surgery in children involving cardiopulmonary bypass (CPB) is associated with an inflammatory response (Laffey, Boylan, and Cheng, 2002; Wan, LeClerc, and Vincent, 1997; Wan and Yim, 1999). Whilst much research in the area of bypass has centred on the role pro-inflammatory cytokines play in the post-operative complications of hypotension, capillary leak and multiorgan failure (Casey, Hauser and others, 1992; Hovels-Gurich, Schumacher and others, 2002; Marano, Garulacan and others, 2000), it is now rare for a patient to die in the early post-operative period from uncontrolled inflammation. The problem facing clinicians is one of delayed morbidity, where patients are ventilator dependant, appear to be immunocompromised, and frequently develop nosocomial infection, followed by multiple organ dysfunction (McBride, Armstrong and others, 1995).

Immune paresis describes a state in critically ill adults following trauma surgery or sepsis, where there is marked suppression in cell-mediated immunity and an associated increased susceptibility to infection. It is thought to be produced endogenously when circumstances elicit a predominantly anti-inflammatory response, or exogenously, when too much immunosuppression is administered. This state is frequently associated with the development of sepsis, unresolving multiple organ dysfunction and late death. Experimental and clinical studies indicate that *immune paresis* involves altered monocyte and T-cell function.

Monocytes from *immunoparalysed* patients show a reduced capacity to secrete TNF- α in response to LPS challenge (Adib-Conquy, Adrie and others, 2000; Haupt, Riese and others, 1998; Heagy, Nieman and others, 2003; Keel, Schregenerberger and others, 1996; Marie, Muret and others, 1998; Yadavalli, Auletta and others, 2001). This has been termed *monocyte deactivation*, and has been reported to persist for three to five days (Haupt, Riese and others, 1998). In addition, this population of monocytes also frequently express reduced levels of MHC Class II expression. Transient *immune paresis* may be protective after an inflammatory insult. However prolonged or extreme deactivation of monocyte function may be harmful as it reduces the host's ability to kill and clear infection.

The findings in Chapter 3 that reduction in monocyte MHC Class II expression preceded the development of sepsis/SIRS by a minimum of 4 days raises the question of whether there *monocyte deactivation* was present in the early post-operative period.

The study discussed in this chapter was designed to investigate the following:

- (i) Does cardiac surgery involving CPB in children alter the production of pro- and anti-inflammatory cytokines in response to lipopolysaccharide (LPS) *ex vivo*?
- (ii) Are high levels of systemic IL-10 following surgery associated with an attenuated response to LPS?
- (iii) Are such cases at increased risk of post-operative sepsis?

5.2 METHODS

5.2.1 Patient selection

This study was carried out over a 15 month period and involved a subgroup of 36 children from the patient group described in chapter 4.

5.2.2 *Ex vivo* whole blood cytokine stimulation

For the purpose of this study, whole blood cultures were used rather than peripheral blood mononuclear cells (PBMC'S) in an effort to mimic the cellular and humoral interactions that occur *in vivo*. One ml of the blood sample was centrifuged immediately at 1500G for 6 minutes, plasma separated and frozen at -70°C , for analysis of baseline plasma cytokine levels (Chapter 4). The remaining whole blood was diluted 1:2 in RPMI 1640 and cultured for 24 hours in the presence or absence of 10ng/mL of purified meningococcal LPS (kind gift from Dr Svein Andersen, EJIVR, Compton UK) at 37°C in atmosphere of 5% CO_2 in air. Following culture, the supernatant was separated by centrifugation and stored at -70°C .

Stored plasma and supernatants were analysed for cytokines using the previously optimised ELISA's. The following cytokines were assessed IL-6, IL-8, TNF- α , IL-10, and IL-1ra. Each patient's samples were analysed together on a single plate. To correct for cytokines already present in the blood before *ex vivo* stimulation with LPS, the amount of each cytokine produced following stimulation was calculated as the difference between cytokine concentrations found after incubation with and without LPS.

5.3 STATISTICS:

Logarithmic transformations were conducted to normalise the distributions of plasma cytokine levels. Levels are presented on a logged scale and values are expressed as mean \pm SEM unless otherwise stated. Comparisons at different time points were performed using the non-parametric Wilcoxon signed-rank test for 2 related samples. A comparison of cytokines between groups over time was made using ANOVA one-way analysis of variance.

5.4 RESULTS:

5.4.1 Dose and time response curves

Prior to commencing this study, dose and time response curves were performed to ascertain the ideal concentration of LPS to be used, and the duration of stimulation (Fig 5.1). The literature varies in the type of endotoxin, dose and duration of stimulation (Dehoux, Hernot and others, 2000;Grundmann, Rensing and others, 2000;Marie, Muret and others, 1998;Munoz, Carlet and others, 1991;Ogata, Okamoto and others, 2000;Randow, Syrbe and others, 1995). The laboratory in which this work was undertaken have an interest in *Neisseria meningitides*, and all results presented are using LPS derived from *N.meningitidis*.

On the basis of results obtained in these preliminary experiments, a concentration of 10 ng/ml of LPS with an incubation period of 24 hours was selected. A stimulation period of 4 hours would have detected maximal TNF- α production but failed to detect the IL-

10 response. Maximal IL-6 and IL-8 responses occurred between 6 – 12 hours (results not shown).

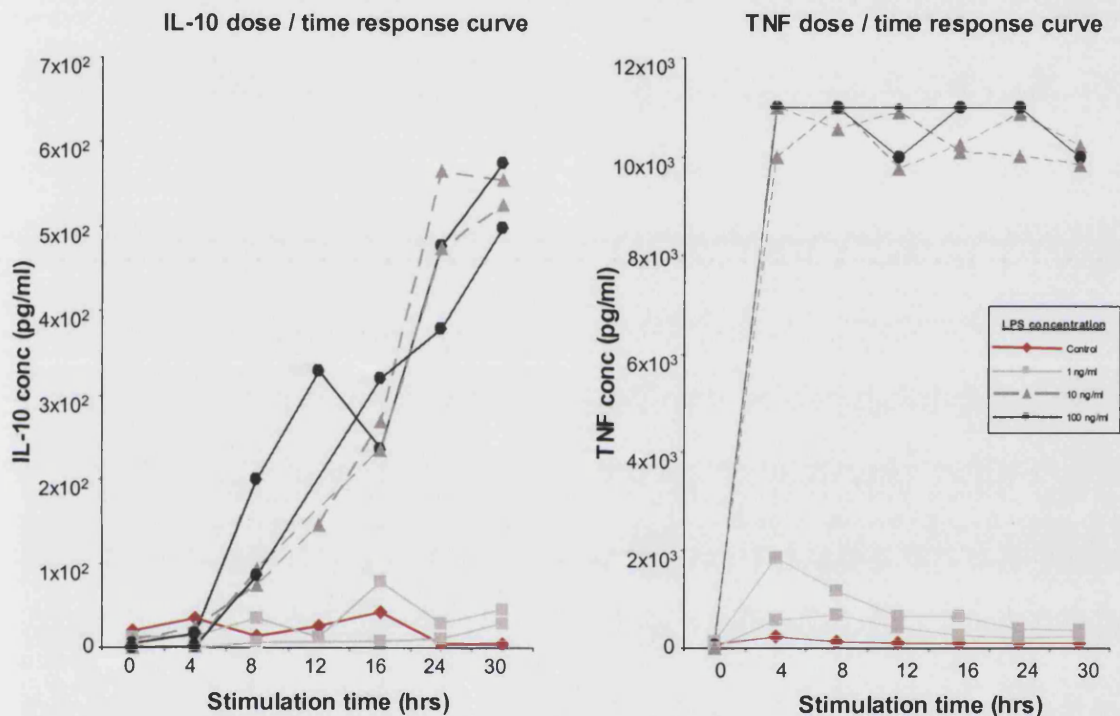


Figure 5.1 IL-10 and TNF- α response to whole blood cultured with varying concentrations of *N.meningitidis* LPS over 30 hours. Experiments were performed with whole blood from 2 adult controls. There were low levels of IL-10 but minimal TNF- α produced in whole blood cultured without LPS. The addition of 1 ng/ml resulted in a small amount of TNF- α production at 4-8 hours, but minimal increase in IL-10 production. There was a dramatic increase in both IL-10 and TNF- α production by whole blood in response to 10 ng/ml LPS. This response was not significantly different to the response seen with 100 ng/ml of LPS. IL-10 production was minimal at 4 hours increasing in a linear fashion to 30 hours. TNF- α production was maximal at 4 hours but appeared to persist at maximal levels for 24 hours. Three out of four of the control samples had decreasing levels of TNF- α when culture times were pushed beyond 24 hours.

5.4.2 Patient Characteristics and Operative Details

Three patients included in the Chapter 4 study were not included in this study because of an inability to process cultured blood the following day. There was no significant difference in the demographics (Mann-Whitney $p>0.6$). Details of the study patients and 3 patients omitted are given in Table 5.1.

Study Patients						
Sex (m:f)	Age (months)	Weight (kg)	CPB Time (mins)	X-clamp time (mins)	Circ arrest Time (mins)	Length of stay (hrs)
21:15	6.5 (9 days – 24 mths)	6.36 (3.1 – 12.9)	110 (49 - 187)	60 (19 - 109)	22 (2 - 24) n=7	89 (24 - 2064)

Omitted Patients						
Sex	Age	Weight (kg)	CPB Time (mins)	X-clamp time (mins)	Circ arrest Time (mins)	Length of stay (hrs)
female	24 days	2.2	99	35	32	100
male	7 months	7.6	52	28	0	21
female	11 months	8.7	60	29	0	65

Table 5.1 Details of 36 study patients and the 3 omitted patients

Results for study patients are presented as mean and ranges.

5.4.3 Outcome

Patients had a median length of stay of 3.7 days (range 1-86), with one third of the patient group requiring greater than 5 days intensive care support. All the patients that developed sepsis/SIRS, or died were included in this study.

5.4.4 *Ex vivo* whole blood cytokine production was reduced in all patients

Pre-operatively whole blood from all patients responded well to LPS stimulation. Whole blood cytokine production to LPS fell significantly for IL-6, IL-8, IL-10 and TNF- α during the period on bypass (Fig 5.2). Maximal hypo-responsiveness to LPS was seen for all these cytokines, on arrival at the intensive care unit ($p < 0.001$). There was an approximate **100 fold** reduction in the ability of whole blood to respond to LPS stimulation at this point. Over the following 48 hours, whole blood IL-10 and TNF- α response to LPS remained significantly below pre-operative levels ($p < 0.005$).

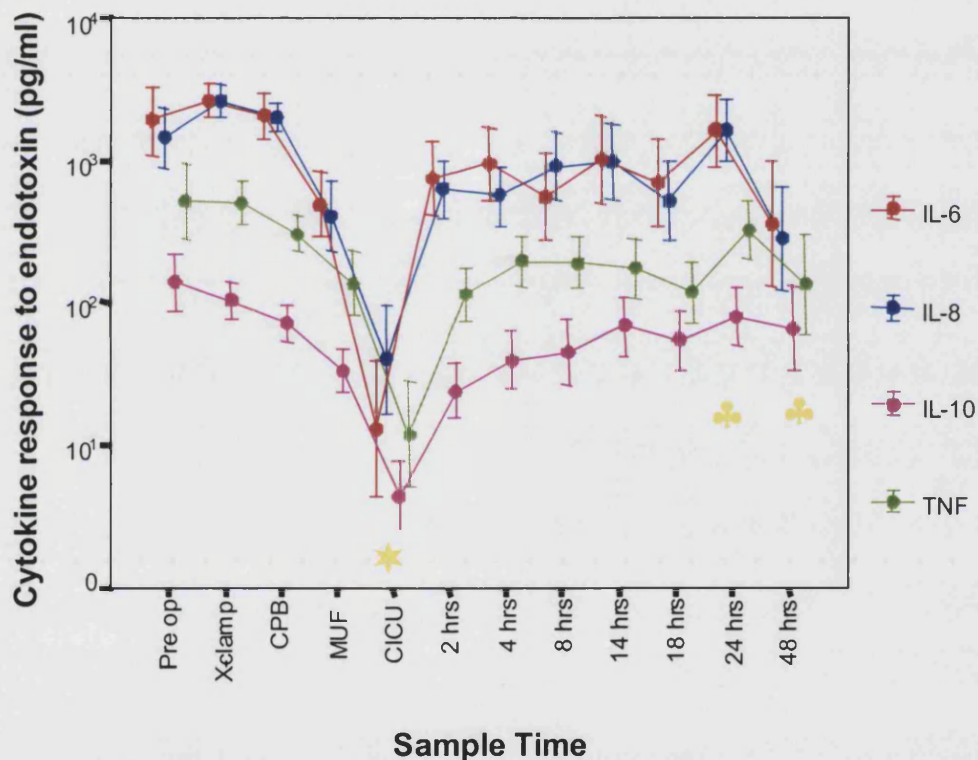


Figure 5.2 *Ex vivo* responsiveness of whole blood to LPS.

Samples of whole blood were drawn from children before during and following cardiac surgery and incubated with and without 10ng/ml of LPS for 24 hours. Plasma supernatants were collected and cytokine concentrations determined by ELISA for interleukin (IL)-6, IL-8, IL-10, and tumour necrosis factor (TNF- α). Cytokine responses to LPS were calculated as [LPS stimulated]-[medium control] at each time point for 36 patients and expressed as mean \pm SEM for the logged values. Clinically relevant time points were chosen (CICU, 24 and 48 hours) and responsiveness was compared to the pre-operative values using Wilcoxon Signed-Rank test. All patients had a significant reduction in cytokine responsiveness to LPS at the end of surgery * $p < 0.001$. This persisted for TNF- α and IL-10 levels at 24 and 48 hours * $p < 0.005$. (Mean background levels of cytokines in whole blood cultured with medium were 130 ± 30 , 590 ± 110 , 37 ± 7 , and 44 ± 7 for IL-6, IL-8, IL-10, and TNF respectively.)

5.4.5 Reduction in cytokine response is not due to a reduction in circulating monocyte numbers in whole blood

One possible explanation for the reduction in whole blood responsiveness to LPS is a fall in the number of circulating monocytes following CPB. However when monocyte counts of whole blood were examined there was no significant reduction in circulating numbers peri-operatively, and an increase in circulating numbers post-operatively when LPS hypo-responsiveness is observed (Fig 5.3).

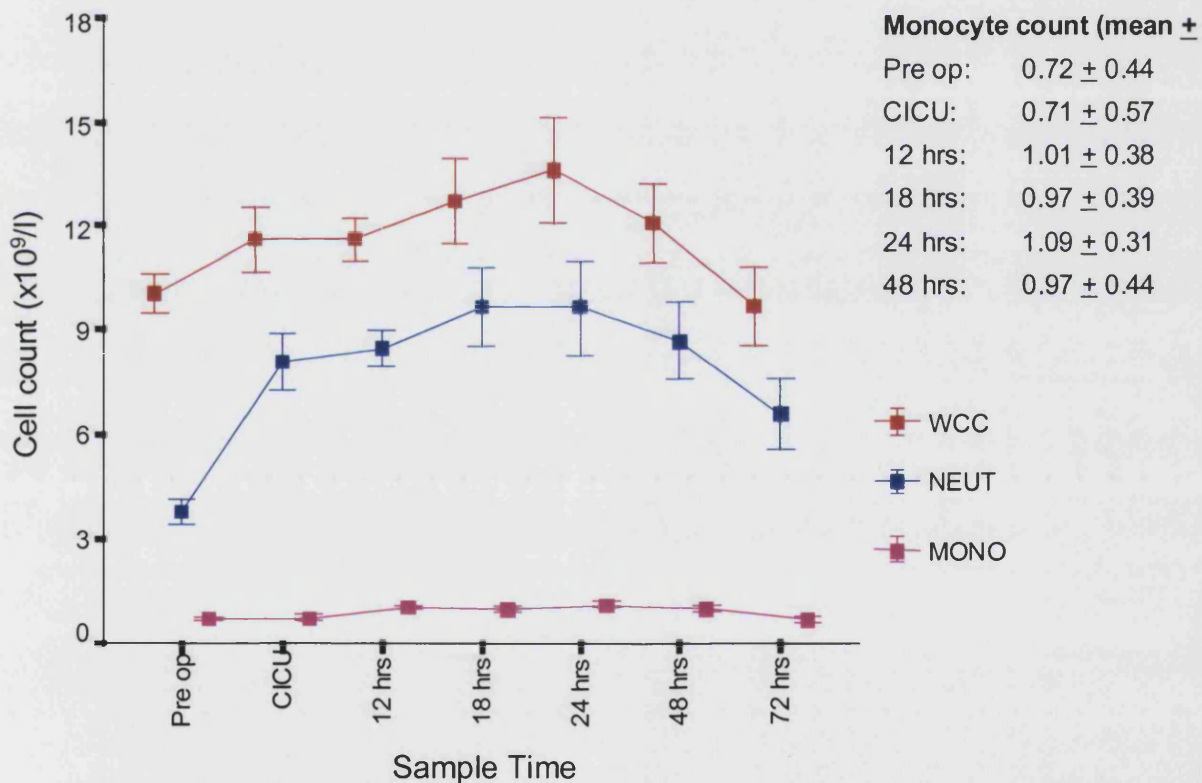


Figure 5.3 Total white cell count, neutrophil and monocyte counts for patient group. Monocyte counts did not significantly change during surgery. Total white cell count, neutrophil and monocyte counts rose in whole blood over the first 24 hours post-operatively before drifting back toward preoperative levels. Monocyte counts were significantly higher than pre-operative levels between 12 – 48 hours post-op (paired t -test $p < 0.05$).

5.4.6 Whole blood hypo-responsiveness precedes the systemic rise in cytokines

There was a steady decline in whole blood TNF- α and IL-10 production in response to LPS through the intra-operative sampling points. This contrasts with the delay seen in systemic cytokines shown in Figure 4.4 of Chapter 4. Whilst whole blood from patients had reduced ability to respond to LPS as early as release of the aortic cross clamp, systemic concentrations of cytokines IL-6, IL-10, and TNF- α were not elevated at this stage. However peak hypo-responsiveness and peak systemic cytokine levels both occurred around the arrival onto CICU.

5.4.7 Patients with high systemic IL-10 response to surgery appeared to be more hypo-responsive to LPS in the post-operative period.

IL-10 cytokine load was calculated as described in Chapter 4 (area under the curve drawn by the systemic IL-10 response for the first 24 hours). Patients with plasma levels > 1 SEM were defined as having a high cytokine load. Five of the 36 patients (14%) were defined as having a high IL-10 load. These patients had a trend towards a greater drop in IL-6 and IL-10 response to LPS stimulation in the post-operative period. Unfortunately 1 of the five patients did not have stimulated samples collected from $t=8$ hrs for technical reasons. Due to the small study size, the apparent greater reduction in responsiveness seen in Figure 5.4 did not reach significance ($p=0.07$). Such a trend however was not seen for IL-8 and TNF- α .

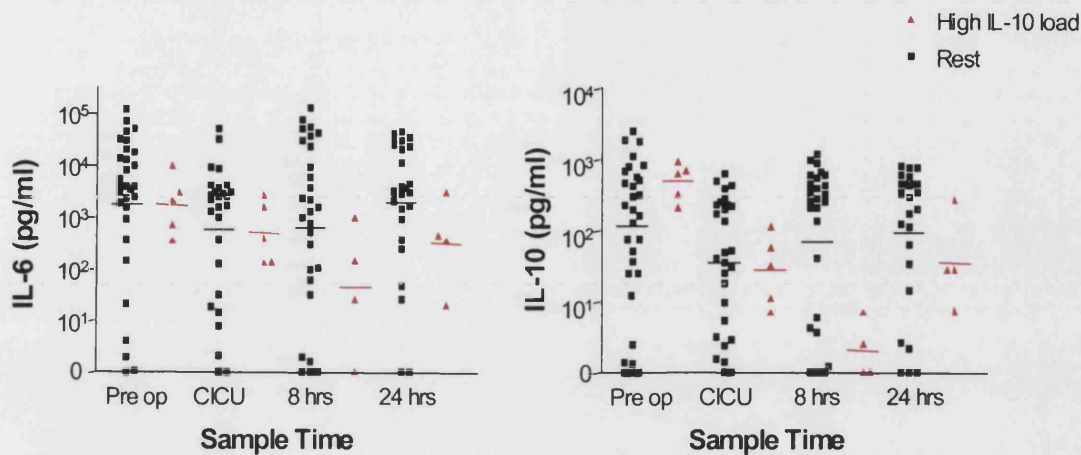


Figure 5.4 Those patients with high circulating IL-10 responses to surgery (cytokine load), appeared to have a greater reduction in cytokine response (IL-6 and IL-10) to whole blood stimulation with LPS. The patient group was divided according to the IL-10 cytokine load produced in response to surgery (Chapter 4). Those patients with a high IL-10 load ($n=5$) appeared less responsive to LPS stimulation (as determined by IL-6 & IL-10 levels) during the first 24 hours post-operatively. One patient in the high IL-10 load group was not sampled at 8 and 24 hours. Horizontal bars represent mean cytokine response for group.

One striking aspect to Figure 5.4 was that patients with a high IL-10 response to surgery (IL-10 load) had a greater pre-operative IL-10 response to LPS stimulation. This difference was not seen when pre-operative levels of the other cytokines were divided according to IL-10 load, and raises the question of the role inter-individual variation in cytokine production.

5.4.8 Patients who developed post-operative complications had reduced cytokine responses to stimulation in the early post-operative period

Those patients who subsequently required prolonged ventilation and intensive care support had significantly lower cytokine responses to stimulation with LPS in the early post-operative period (ANOVA $p < 0.05$ for all cytokines) (Fig 5.5).

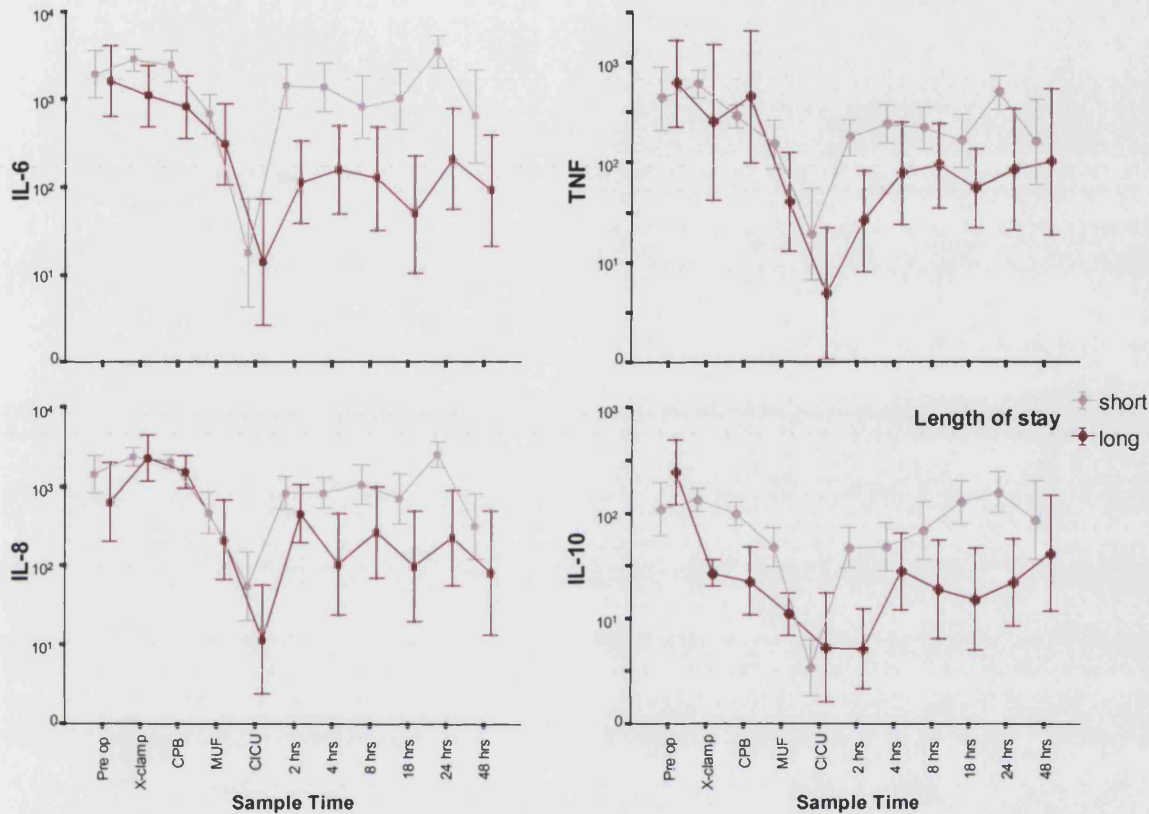


Figure 5.5 Long stay patients were more hypo-responsive to LPS stimulation in the post-operative period than short stay patients.

The patient group was divided according to whether their stay in CICU was short (≤ 5 days) or long (> 5 days). Those patients who subsequently required prolonged support were less responsive to LPS stimulation in the early post-operative period.

There was also a trend toward a lower cytokine response to stimulation in the 4 patients who developed sepsis or SIRS. This reached significance for IL-10 and TNF- α responses when sepsis/SIRS and deaths ($n=5$) were considered together (ANOVA $p = 0.026$ and $p = 0.017$ respectively) (Fig 5.6).

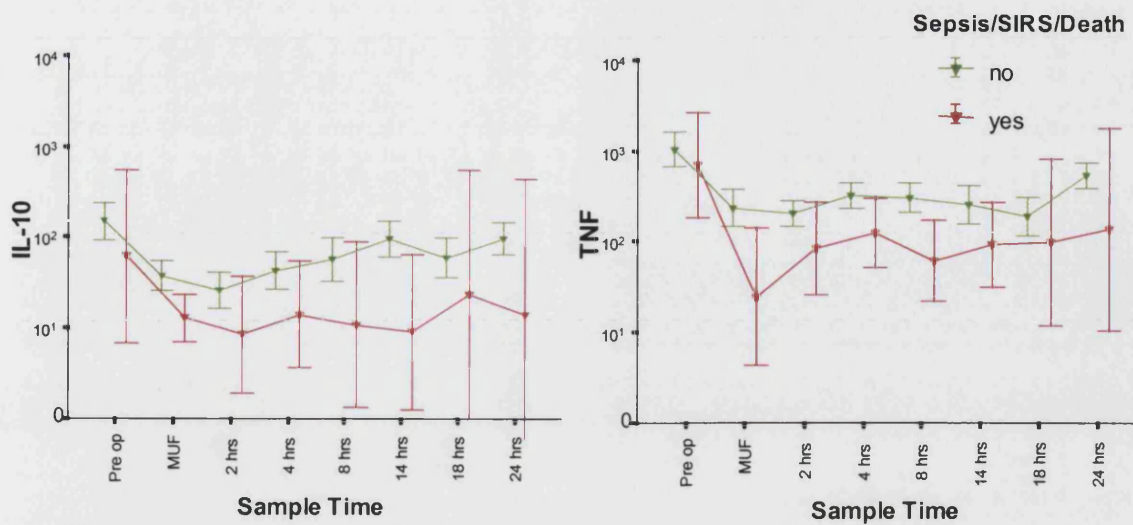


Figure 5.6 Patients who developed sepsis/SIRS or died following surgery had a more profound inability to respond to LPS.

36 patients were examined for LPS hypo-responsiveness following cardiac surgery involving CPB. Three patients developed sepsis, 1 patient fulfilled the criteria for SIRS, and 2 patients died (1 was also septic). Examination of these 5 patients' cytokine responses to LPS revealed lower cytokine responses for IL-6, IL-8, IL-10, and TNF- α . This reached significance for IL-10 and TNF- α when the 3 outcomes were combined (ANOVA $p = 0.026$ and 0.017 respectively).

5.4.9 Timing of the whole blood hypo-responsiveness coincides with the reduction in monocyte MHC Class II expression

The reduced ability of whole blood to respond to LPS stimulation peri-operatively parallels in time the reduction in surface expression of MHC Class II on monocytes described in chapter 4. Figure 5.7 shows graphs for each cytokine (mean \pm SEM), where the patient's responsiveness to stimulation at each sampling time has been expressed as a percentage of the pre-operative whole blood response. Monocyte MHC Class II expression (%) is superimposed. Neither the MHC Class II expression nor the whole blood responsiveness to LPS had returned to pre-operative levels at the end of this study (Fig 5.7).

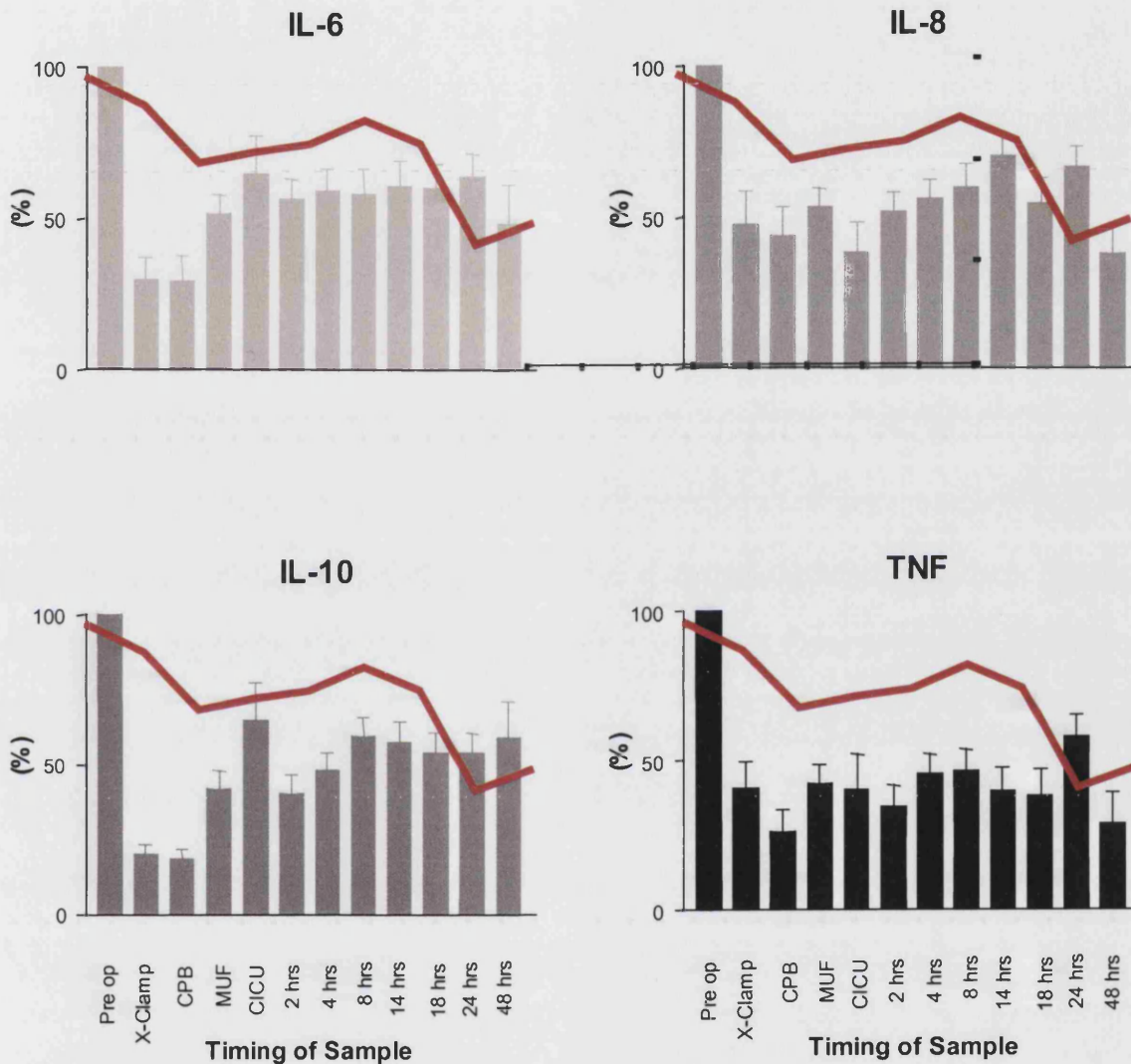


Figure 5.7 MHC Class II expression (%) on circulating monocytes superimposed on whole blood cytokine responsiveness. MHC Class II expression (— median).

5.4.10 Whole blood hypo-responsiveness to LPS stimulation following CPB does not occur for all cytokines

Whilst whole blood cultures stimulated with LPS from patients following cardiac surgery had a significant reduction in the ability to produce IL-6, -8, -10 and TNF- α this was not true for all cytokines. Figure 5.8 shows that the same patient cultures continued to produce high levels of IL-1ra through surgery and over the first 48 hours following surgery. As these were whole blood cultures, the possible role of neutrophils in maintaining the production of IL-1ra has not been excluded.

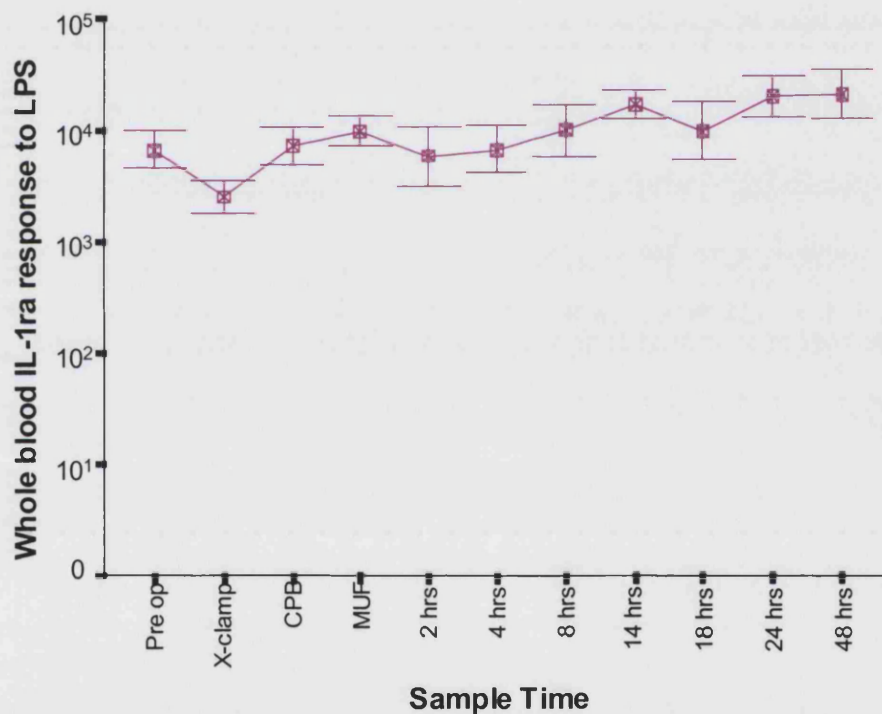


Figure 5.8 Whole blood IL-1ra response to LPS stimulation. There was no change in whole blood IL-1ra production in response to LPS through the peri-operative period.

5.5 DISCUSSION

The results presented in this chapter show that children undergoing cardiac surgery involving cardiopulmonary bypass enter a period of LPS hypo-responsiveness in the peri-operative period. This hypo-responsiveness was profound and prolonged, and coincided with the reduction in surface MHC Class II expression documented in chapter 4. The specific combination of reduced MHC Class II and an inability of whole blood to produce TNF- α on LPS stimulation has been suggested as an objective clinical measure of '*immune paresis*' (Docke, Randow and others, 1997; Volk, Reinke and others, 1996; Volk, Reinke, and Docke, 1999). The hypo-responsiveness to LPS reported here is most likely reflecting an inability of monocyte to respond, as these are the major cytokine secreting cell in whole blood. This inability of monocytes to respond to LPS stimulation with the production of pro-inflammatory cytokines has been termed *monocyte deactivation*.

The timing of cytokine production may be critical to the subsequent induction of LPS hypo-responsiveness. In chapter 4, high levels of all cytokines were detected systemically within the first two hours of surgery. This corresponds to the maximal period of cytokine hypo-responsiveness. The reasons for this are unclear. If it is assumed that monocytes are the main producers of these cytokines in whole blood, then one possibility is that circulating monocytes become '*exhausted*' and cannot mount a response to a secondary challenge. However the persistence of the IL-1ra response to stimulation, which occurred at levels comparable to those measured pre-operatively, argues against such a hypothesis although selective '*exhaustion*' is still a possibility. In addition, IL-1 and IL-1ra can be produced by neutrophils to a greater extent than the other cytokines examined. The persistence of an IL-1ra response may merely reflect ongoing secretion of IL-1ra by neutrophils in response to the LPS stimulation. Additional experiments involving stimulation of both whole blood and isolated monocytes are required to investigate this observation further.

This observation that deactivation is selective has also been reported by Randow et al (1995) (Randow, Syrbe and others, 1995). In this study Randow cultured PBMC's from healthy donors with first a low and then a high dose of *Escherichia coli* endotoxin over two 24 hour periods. Whilst IL-10 and TNF- α production were greatly decreased

following the second period of culture, IL-1ra production was not. He went on to confirm these findings in stimulation experiments involving PBMC's isolated from septic adult patients. Selective tolerance of monocytes has not been reported in trauma or surgical patients to date.

The mechanism underlying the selective deactivation of monocytes is not completely understood. Most of the work has been done in *in vitro* cell cultures using LPS - induced tolerance, and not trauma. Initially, using an endotoxin tolerance model developed with mouse peritoneal macrophages it was felt that tolerance may have been due to a reduction in surface Toll-like receptor-4 (TLR4) expression in response to exposure to LPS (Nomura, Akashi and others, 2000;Takeda and Akira, 2001), but this has not been a consistent finding (Medvedev, Kopydlowski, and Vogel, 2000;Medvedev, Lentschat and others, 2002). Whilst LPS is recognised to bind to CD14, it signals through the TLR4 molecule via the myeloid differentiation factor 88 (MyD88) / IL-1R-associated kinase 1 (IRAK-1) pathway to activate NF- κ B resulting in TNF- α production (Adib-Conquy and Cavaillon, 2002;Jacinto, Hartung and others, 2002). More recently a decrease in TLR4-MyD88 complex formation with subsequent impairment of IRAK-1 activity has been shown to occur in LPS-tolerant monocytes (Jacinto, Hartung and others, 2002;Medvedev, Lentschat and others, 2002). Whilst it is most likely that tolerance involves alteration of distinct TLR signalling components, neither of these findings addresses the fact that LPS-tolerant monocytes can still respond to further LPS stimulation by expressing other genes and proteins.

In vitro studies would suggest that the aetiology of *immune paresis* is in part due to high circulating levels of IL-10 (Randow, Syrbe and others, 1995). Certainly IL-10 was shown in Chapter 4 to be elevated in this patient group by the end of surgery. In addition those patients with the greatest IL-10 response to surgery (IL-10 load), had a more marked hypo-responsiveness to endotoxin.

The onset of hypo-responsiveness appeared to precede the systemic rise in circulating cytokines, in particular IL-10. Many patients had a 50% reduction in cytokine response to endotoxin by the time the aortic cross-clamp was release, suggesting that factors other than IL-10 may play a role. This concept is supported by work carried out in isolated CPB circuits where hypo-responsiveness is seen in the absence of circulating

IL-10 (Dehoux, Hernot and others, 2000;McBride, Armstrong and others, 1995). Dehoux et al (2000) (Dehoux, Hernot and others, 2000) showed that within 30 minutes of passing control whole blood through a sham bypass circuit there was reduced IL-6, IL-10, and TNF- α response to LPS stimulation. This hypo-responsiveness persisted whilst the blood circulated in contact with the circuit (up to 180 min), and occurred despite the absence of detectable levels of IL-10 or endotoxin.

Circulating levels of IL-10 may contribute to the ongoing *immune paresis*, with high systemic levels of IL-10 appearing to further suppress whole blood IL-6 and IL-10 response to endotoxin in this study. Whilst it has been possible to show an association between poor outcome and reduced MHC Class II expression and reduced capacity of whole blood to respond to endotoxin, it has not been possible to show a direct correlation between high IL-10 plasma load, long stay and the development of sepsis or SIRS.

The development of *immune paresis* is felt to be a risk factor for the subsequent development of nosocomial infection and post-operative complications, and represents dysregulation of the immune balance toward an anti-inflammatory state (CARS). In this small study group it was possible to show that patients who required prolonged intensive care support, or who developed sepsis/SIRS had a greater degree of whole blood hypo-responsiveness in the immediate post-operative period.

From *in-vitro* and *in-vivo* studies it appears that both MHC Class II expression and reduced TNF- α production can be reversed by immunomodulation with interferon-gamma (IFN- γ) or granulocyte-macrophage colony-stimulating factor (GM-CSF), and that restoration of MHC Class II expression is associated with improved outcome (Docke, Randow and others, 1997;Flohe, Borgermann and others, 1999;Kox, Bone and others, 1997).

Interleukin-10 can be shown to cause suppression of pro-inflammatory cytokine secretion by monocytes, and reduced surface expression of Class II molecules with corresponding impaired antigen presentation (Randow, Syrbe and others, 1995). However there are potentially two problems with the hypothesis that IL-10 is the primary mediator behind both LPS hypo-responsiveness and reduced monocyte MHC

Class II surface expression. IL-10 secretion inhibits its own production in addition to that pro-inflammatory cytokines, so any monocyte deactivation may be short lived or cyclical in nature. Secondly, this study showed that LPS hypo-responsiveness precedes the systemic rise in IL-10 following CPB in children. It is important to recognise that plasma levels of IL-10 may not reflect levels in the immediate vicinity of tissue/cells.

A possible alternative explanation may be that there are 2 phases to the hypo-responsiveness. The first is induced by anaesthesia, interaction of patient's blood with the bypass circuit, or as a direct result of the trauma of surgery. Then, circulating inflammatory mediators maintain the later hypo-responsiveness and reduction in Class II surface expression, in particular, high levels of IL-10.

It is now known that LPS binding to TLR4 induces a wide array of gene expressions, including the TNF- α and COX-2 gene, initiating the septic response. However these pro-inflammatory genes are rapidly and continuously down-regulated soon after initiation. Whilst the anti-inflammatory genes are also activated early in sepsis, they do not appear to be switched off. This differential expression of pro- and anti-inflammatory genes can potentially lead to a state of immunosuppression.

Alternatively, a factor we have not examined in this study may be responsible for both the hypo-responsiveness and the high IL-10 load. Such a factor could include humoral mediators rapidly released with the onset of CPB, mechanically induced damage, or change in cellular function following contact of blood with bypass circuit.

Other mediators which may be considered in LPS hypo-responsiveness following CPB include transforming growth factor- β (TGF), endotoxin, opioids, prostaglandins, and glucocorticoids. TGF- β is an immunoinhibiting cytokine and has been shown to induce LPS hypo-responsiveness in PBMC's in vitro. Addition of neutralising anti-TGF- β monoclonal antibodies prevents this induction (Randow, Syrbe and others, 1995). TGF- β was not measured in this study or that reported in Chapter 4. Like IL-10, TGF- β levels have been shown to be raised at the end of CPB in adult patients, but quickly return to pre-operative levels by 6 hours post-op (Sablitzki, Welters and others, 1997). Based on this kinetics, TGF would not explain the persistent hypo-responsiveness seen in these results and does not augment plasma IL-10 levels.

Endotoxin levels have been associated with adverse outcomes following cardiac surgery. Endotoxin and anti-endotoxin core antibodies have been documented in the plasma of adults undergoing cardiac surgery, peaking 30 minutes postoperatively and preceding plasma peaks of IL-6 and IL-8 (Neuhof, Wendling and others, 2001; Rothenburger, Soeparwata and others, 2001). The primary source of endotoxin has traditionally been perceived to be gut translocation, but it has also been found in pooled pericardial blood (Spanier, Tector and others, 2000). Whilst endotoxin would explain both LPS hypo-responsiveness and high plasma IL-10 levels *in vivo*, endotoxin is absent in in-vitro studies involving isolated bypass circuits where LPS hypo-responsiveness has occurred (Dehoux, Hernot and others, 2000; McBride, Armstrong and others, 1995).

Randomised adult CPB studies using high vs. low dose opioid anaesthesia showed no difference in pro- and anti-inflammatory cytokine response (Brix-Christensen, Tonnesen and others, 1998). The kinetics of LPS hypo-responsiveness presented above and in other studies has shown an onset of hypo-responsiveness following CPB, not anaesthetic induction. In addition, the presence of fentanyl to whole blood cultures from healthy volunteers does not alter *ex-vivo* LPS-induced cytokine production (Larsen, Hoff and others, 1998; Paquin, 1996), and LPS hypo-responsiveness has occurred when blood was passed through an isolated bypass circuit in the absence of opioids (Dehoux, Hernot and others, 2000).

Following cardiac surgery, there is an increase in both prostaglandin E2 and glucocorticoids. These endogenous mediators are known to inhibit LPS-induced production of cytokines (Scales, Chensue and others, 1989; van der, Jansen and others, 1994). However it is unlikely that endogenous cortisol is responsible for the LPS hypo-responsiveness seen in this patient group as adult studies suggest that anaesthesia used for CPB blocks the neuroendocrine stress response, and the rise in systemic cortisol occurs on arrival onto the intensive care unit (Roth-Isigkeit, Brechmann and others, 1998; Roth-Isigkeit, Dibbelt, and Schmucker, 2000). This time course would be out of keeping with the intra-operative hypo-responsiveness reported here.

Steroids have been used in numerous adult clinical studies involving cardiac surgery. Whilst results are not always consistent, it appears that steroids reduce systemic pro-inflammatory cytokine levels and augment the IL-10 response (Chaney, 2002;Fillinger, Rassias and others, 2002;Harig, Feyrer and others, 1999;Tabardel, Duchateau and others, 1996). However no patient in this study received exogenous glucocorticoids during theatre.

In summary, all children entered a period of monocyte hypo-responsiveness following CPB marked by a reduction in surface expression of MHC Class II and reduced ability to mount a cytokine response to LPS stimulation. Whilst those patients who required prolonged support, developed sepsis/SIRS or died had a more profound hypo-responsiveness, and a greater hypo-responsiveness was seen in those patients with a high IL-10 load, IL-10 alone does not appear to be the sole mediator. Long stay and septic patients did not have significantly higher levels of IL-10, and hypo-responsiveness preceded the systemic rise in IL-10.

It is clear that environmental factors can and probably do influence the outcome and cytokine dynamics of children undergoing cardiac bypass. However it is also possible that genetic factors may be important and this is addressed in the next chapter.

CHAPTER 6

Genetic polymorphisms: Susceptibility or resistance to inflammatory insult

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6.1 INTRODUCTION:

“Gene isolation provides the best hope for understanding human disease at its most fundamental level. Knowledge about genetic control of cellular functions will underpin future strategies to prevent or treat disease phenotypes.”

Francis Collins Shattuck lecture: medical and societal consequences of the Human Genome Project (1999).

The outcome for any individual after major surgery is determined by the nature and magnitude of both the peri-operative challenge and patient response. Each of these components is itself multifaceted. It has become increasingly clear that the genetic constitution of the patient plays a powerful role in the transduction of the environmental stimulus and the nature of subject response.

Several studies have highlighted the inter-individual variation in cytokine responses to endotoxin (Lowe, Galley and others, 2003 ;Molvig, Baek and others, 1988;Westendorp, Langermans and others, 1995 ;Westendorp, Langermans and others, 1997). Recent studies in both adults and children have suggested that cytokine responses are under significant genetic control (Andreotti, Porto and others, 2002 ;Brull, Montgomery and others, 2001). In the results presented thus far, two sections have raised the question ‘Is an individual genetically predisposed to respond to an inflammatory insult in a particular way?’ The first was in chapter 3 (section 3.4.13) where the MHC Class II intensity on monocytes (mfi) preoperatively was significantly lower in those children who subsequently required prolonged support, developed sepsis and/or SIRS. The second was in Chapter 5 (section 5.4.7), where it was found that children who were high IL-10 producers in response to surgery, had a significantly greater degree of hypo-responsiveness to secondary challenge with endotoxin. Not surprisingly, this subgroup of children had a much greater IL-10 response to endotoxin stimulation pre-operatively, before becoming profoundly hypo-responsive post-operatively (Fig 5.4).

The concept that a child could be genetically predisposed to do poorly following the inflammatory insult of CPB is very attractive clinically. At present every effort is made to accurately stratify each patient according to operative risk. However the host response is still the greatest unknown.

The aim of this chapter was to investigate the relative role of key functional cytokine polymorphisms on the cytokine response and outcome of children undergoing cardiopulmonary bypass. The hypothesis was that children with a promoter polymorphism that resulted in increased systemic levels of IL-6 or TNF- α would be more likely to have a complicated postoperative period. Cytokines chosen were IL-6 and TNF- α because of repeated implication in the pathogenesis of myocardial dysfunction and ischaemia-reperfusion injury following cardiac surgery, and IL-10 because of its recently highlighted role in attaining immune balance. Whilst a number of polymorphisms have been reported in each of these cytokine promoters, IL-6 -174 (G/C), TNF- α -308 (A/G), and IL-10 (-1082, -819, -592) have been consistently shown to have a functional effect on protein levels.

6.2. METHODS

6.2.1 Patient Selection

Any patient undergoing cardiac surgery involving cardiopulmonary bypass over an 18 month period was eligible for entry into this study.

6.2.2 Blood sampling for genotyping and measurement of cytokines

Each patient had 1-2 ml of whole blood collected into EDTA pre-operatively as described in Section 2.3.6 of the Chapter 2. In an initial subgroup of 42 patients, plasma cytokine levels were measured pre and post-operatively, and over the following 48 hours.

6.2.3 Heteroduplex Genotyping

Initially a heteroduplex method for genotyping was examined for detection of the IL-10 promoter polymorphism. This method involved generation of a universal heteroduplex generator (UHG). The UHG was a section of the genomic DNA of interest which contained a small polypurine insert adjacent to the site of the polymorphism. Both the UHG and the genomic DNA were amplified using primers designed to span the area containing the polymorphism. Equal amounts of both UHG and PCR product were then combined and denatured. When the denatured products were allowed to slowly renature, they formed DNA duplexes. Those duplexes with the same sequence in both strands, (homoduplexes) electrophoresed more quickly than those duplexes with a base pair mismatch (heteroduplex) (Bhattacharyya and Lilley, 1989).

6.2.3.1 DNA extraction and standardisation

DNA was extracted and standardised as described in Section 2.3.6.1.

6.2.3.2 PCR Primers

Table 6.1 details the primer pairs that were used for PCR amplification of genomic DNA and the heteroduplex generators:

Polymorphism	Oligonucleotide Sequence 5'-3' (Forward / Reverse)
IL-10 -1082 G/A	5'-AAT CCA AGA CAA CAC TAC TAA GGC-3' 5'-CTG GAT AGG AGG TCC CTT AC-3'
IL-10 -819 C/T	5'-TAC AGT AGG GTG AGG AAA CC-3' 5'-GGT AGT GCT CAC CAT GAC CC-3'
IL-10 -592 C/A	5'-GAA ATC GGG GTA AAG GAG CC-3' 5'-AGT TCC CAA GCA GCC CTT CC-3'

Table 6.1 Primer pairs for amplification of DNA around the IL-10 promoter area of interest (Morse, Olomolaiye and others, 1999).

6.2.3.3 Universal Heteroduplex Generators (UHG)

UHG's were synthesised as single long oligonucleotides (kindly provided by Dr N Wood, University of Bristol, Bristol, UK) (Table 6.2).

IL-10 -1082 UHG	5'-AAT CCA AGA CAA CAC TAC TAA GGC TTC TTT GGG AAA AAA AGG GGA AGT AGG GAT AGG TAA GAG GAA AGT AAG GGA CCT CCT ATC CAG-3'
IL-10 -819 UHG	5'-TAC AGT AGG GTG AGG AAA CCA AAT TCT CAG TTG GCA CTG GTG TAC CCT TGT ACA GGT GAT GTA ACA AAA AAT CTC TGT GCC TCA GTT TGC TCA CTA TAA AAT AGA GAC GGT AGG GGT CAT GGT GAG CAC TAC C-3'
IL-10 -592 UHG	5'-GAA ATC GGG GTA AAG GAG CCT GGA ACA CAT CCT GTG ACC CCG CCT GTC AAA ACT GTA GGA AGC CAG TCT CTG GAA AGT AAA ATG GAA GGG CTG CTT GGG AAC T-3'

Table 6.2 Nucleotide sequences for the UHG molecules

6.2.3.4 Optimisation of PCR and Heteroduplexing

The PCR for all 3 promoter polymorphisms were optimised for MgCl₂ concentration and temperature (1.5mM and 57°C respectively) using control DNA. Extremely dilute amounts of UHG were used in order to avoid smearing from excess product.

Equal volumes of genomic DNA and UHG product were mixed, denatured at 95°C for 5 min and allowed to cool slowly from 95°C to 37°C over 30 minutes. Diffusion through 15 and 20% vertical non-denaturing polyacrylamide gels was examined using varying currents. Low currents for long periods were required to achieve even diffusion of samples. Optimal electrophoresis conditions were found to be 6 hours at 160V in 20%

polyacrylamide gel. Gels were stained for 5 min in 1x TBE containing 0.5µg/ml ethidium bromide, and examined using a UV transilluminator.

6.2.4 Microtitre Array Diagonal Gel Electrophoresis (MADGE)

A detailed description of the method used in this chapter is presented in section 2.3.6 of Chapter 2. Detection of promoter polymorphism for IL-6 -174 (G/C) had been previously established using Microtitre Array Diagonal Gel Electrophoresis (MADGE) by the Cardiovascular Genetic laboratory at The Rayne Institute, University College London (Brull, Montgomery and others, 2001 ;Fishman, Faulds and others, 1998). After optimisation of the IL-6 method for this patient group, the technique was extended and optimised for the detection of the IL-10 -1082 (G/A), -819 (C/T), -592 (C/A) and TNF- α -308 (G/A) promoter polymorphisms. Appropriate digestion enzymes for these promoter polymorphisms were obtained from the literature and conditions optimised to give clear digest bands (Turner, Williams and others, 1997).

6.3 STATISTICAL ANALYSIS

Allele frequencies were estimated by gene counting. The goodness of fit between observed and expected allele frequency was statistically tested using a χ^2 test. Plasma cytokine levels are presented on a log scale and comparisons between data sets was made using Kruskal-Wallis. Variations in plasma cytokine levels between genotype groups were examined by ANOVA. Multiple comparisons were adjusted for using Bonferroni corrections and a *p*-value of <0.05 was considered statistically significant. A 2-tailed t-test for equality of means was utilised when comparing duration of ventilation and length of stay between genotypes.

6.4 RESULTS

6.4.1 Heteroduplex genotyping for IL-10 promoter polymorphisms

The heteroduplex method enabled differentiation of the three IL-10 polymorphisms (Fig 6.1). However considerable time was required for optimisation and it was therefore felt that time would be prohibitive for optimisation of this method for the other cytokine polymorphisms. An alternative system was therefore explored.

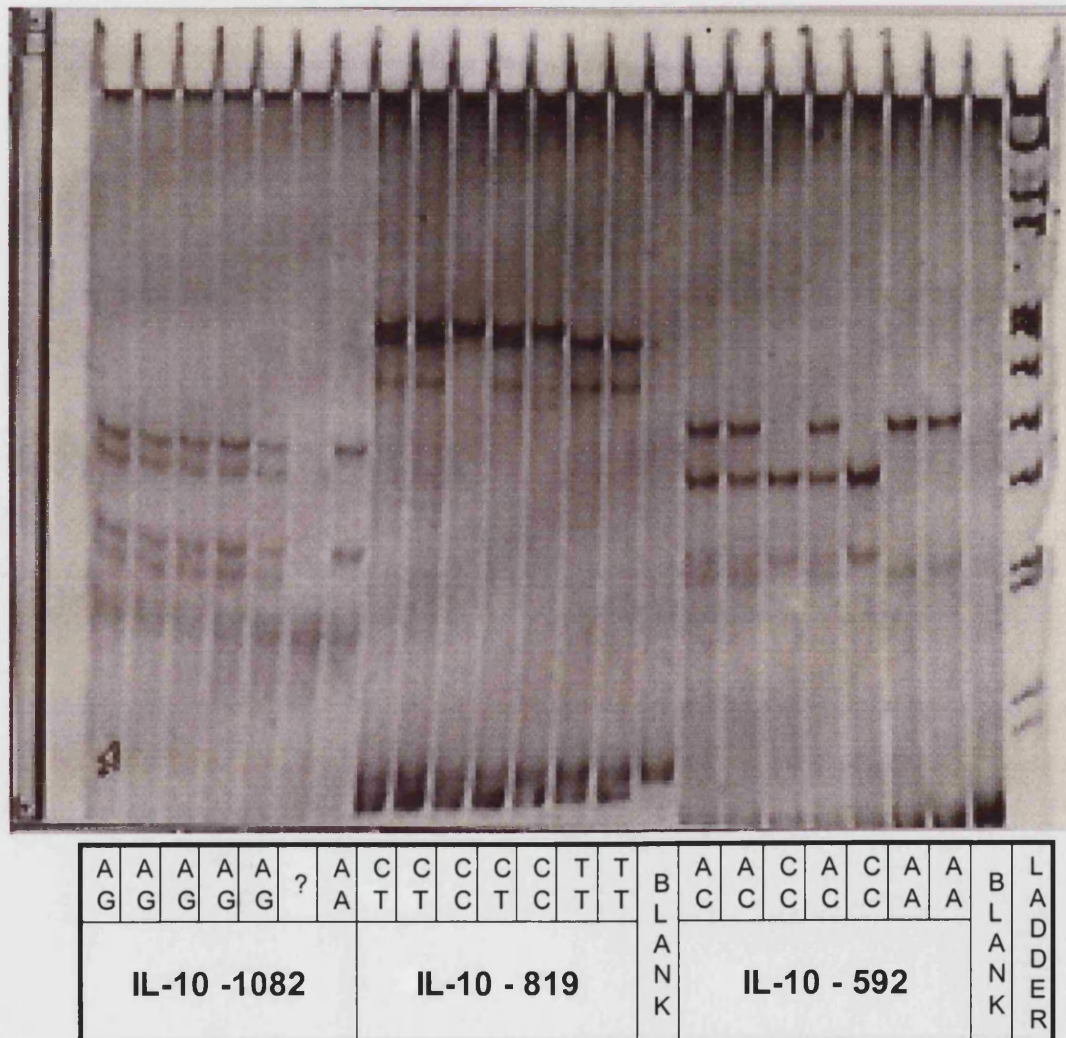


Figure 6.1 Image of a 20% polyacrylamide gel of the IL-10 promoter polymorphisms. This gel contained 7 samples examined for the 3 IL-10 promoter polymorphisms. A key to the bands is drawn under each sample on the gel. On this 24 well gel there were 2 blanks and one drop out (6th well from the left). The 1st sample (from the left), would be read as a GCC/ATA haplotype, the 5th sample as GCC/ACC, and the last control an ATA homozygote

6.4.2 Development of MADGE and optimisation of the IL-10 -1082, -819, -592, and the TNF- α -308 promoter polymorphisms

Primer pairs to amplify the appropriate region of the promoters of interest were obtained from the literature (Morse, Olomolaiye and others, 1999). Optimisation of PCR conditions was performed in a 96 well plate combining a temperature gradient on the Thermocycler with a $MgCl_2$ gradient in the PCR mix. Optimisation was performed on patient samples with duplicates of each condition. Figure 6.2 is a photograph of a gel optimising the TNF- α -308 promoter polymorphism. Extra bands are seen in the lower left corner, where low temperatures and high magnesium conditions (for these primers) were present. This contrast with the top right region of the gel, where high temperatures and low magnesium concentrations give clear single bands of product. Well C12 – G12 were blank in this gel. In this case optimal conditions for this PCR were chosen to be 57°C with 1.5 mM concentration of $MgCl_2$. After optimisation the product was checked on an agarose gel against an appropriate ladder to ensure it was of the correct size.

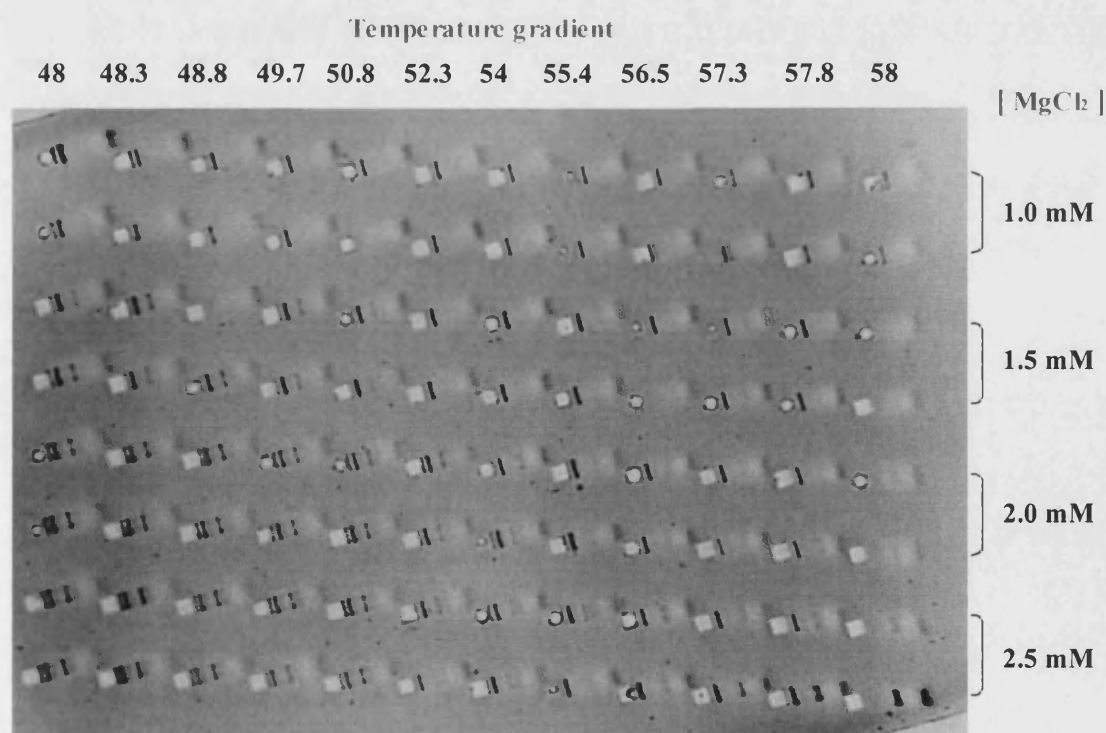


Figure 6.2 Optimization $MgCl_2$ and annealing temperature for TNF- α -308 (G/A) promoter polymorphism PCR. Each PCR was optimized for magnesium concentration and annealing temperature to minimize non-specific binding and primer dimmer. Conditions involving the highest temperature and lowest magnesium concentration producing a clear band of product of the appropriate size were chosen.

A literature search was performed to identify restriction enzymes that cut at or near the site of the promoter polymorphism of interest. Ideally the enzyme chosen would cut the common sequence. This, in combination with the presence of a positive control on each plate, ensures that the operator can be certain the digest has worked even in rare polymorphisms. Figure 6.3 demonstrates the restriction enzyme EcoN1 used for the IL-10 -1082 promoter polymorphism.

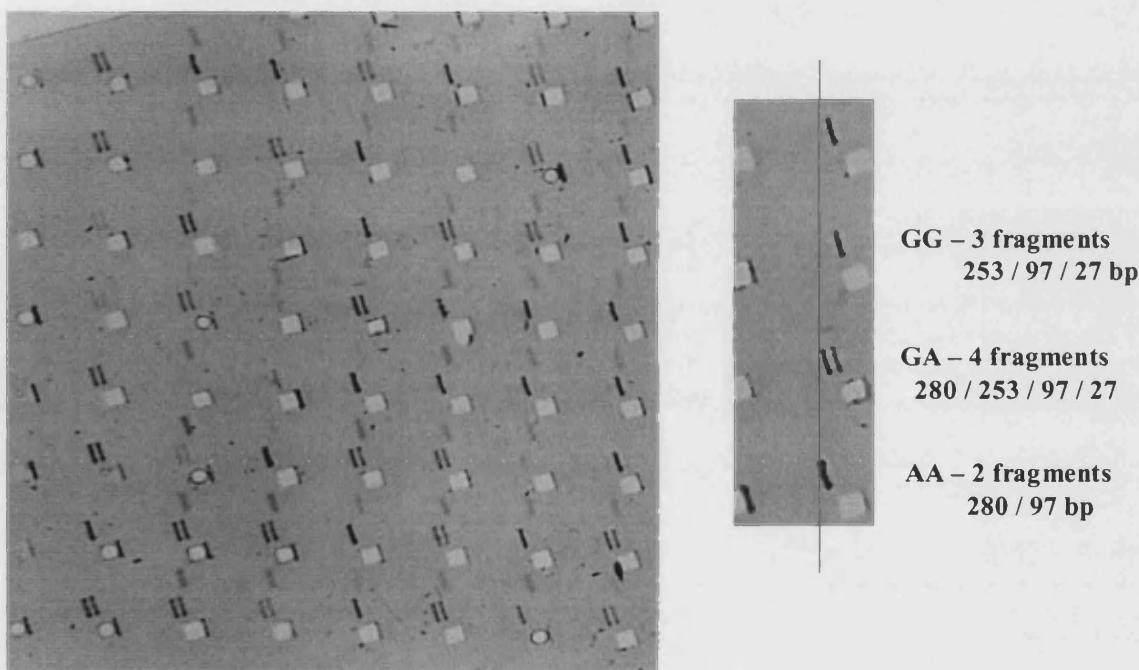


Figure 6.3 Microtitre Array Diagonal Gel Electrophoresis (MADGE) for IL-10 (-1082 G/A) promoter polymorphism. Following digestion with EcoN1 overnight at 37°C, 5µl of the PCR product was added to 2µl of formamide dye and loaded onto a 7.5% polyacrylamide gel. The gel was electrophoresed at 100V to separate digest products and visualised using UVP Gel Documentation System. The digestion enzyme EcoN1 cuts the PCR product 3 times if guanine is present at the -308 position, but only twice if there has been a base change to adenine.

6.4.3 Patient Characteristics and Operative Details

Over the 18 months of study recruitment, 450 children underwent elective cardiac repair involving cardiopulmonary bypass. Two hundred and thirty of these were randomly approached (depending on availability of the author to recruit). All but three agreed to participate. Complete phenotypic and genotypic data was available on 219 patients. Demographics and operative details for the whole study cohort are shown in Table 6.3. In addition the same details are presented for each of the promoter polymorphisms examined. There was no statistically significant difference in demographics and operative details within any of the cytokine promoter genotypes, nor between a particular genotype and the overall study group.

		IL-6 (-174)			IL-10 (-1082, -819, -592)			TNF- α (-308)		
	All (n=219)	GG (n=87)	GC (n=104)	CC (n=28)	GCC/GCC (n=45)	GCC/- (n=104)	-/- (n=70)	GG (n=142)	GA (n=71)	AA (n=6)
Age (mths)	7.4 (2.6 - 31)	6.5 (0.9 - 20)	8.1 (2.7 - 44)	8.7 (3.6 - 23)	8.8 (3 - 32)	8.2 (2.7 - 31)	5.2 (2.1 - 34)	7.4 (2 - 33)	7.2 (3.2 - 18)	33.8 (2.1 - 111)
Male	120 (55%)	49 (56%)	57 (55%)	14 (50%)	17 (38%)	57 (55%)	46 (66%)	88 (62%)	28 (40%)	4 (67%)
Weight (kg)	7 \pm (4-10)	5 (4-9)	7 (4-17)	8 (5-10)	5 (4-9)	7 (4-10)	8 (5-14)	6 (4-12)	7 (5-9)	10 (5-40)
Operative Group (Jenkins Criteria)										
1	17 (8%)	8 (10%)	8 (8%)	1 (4%)	5 (9%)	9 (9%)	3 (4%)	11 (8%)	6 (8%)	0 (0%)
2	85 (39%)	32 (37%)	38 (37%)	15 (53%)	16 (36%)	42 (40%)	27 (38%)	58 (41%)	24 (34%)	3 (50%)
3	77 (34%)	32 (36%)	36 (34%)	9 (32%)	16 (36%)	32 (31%)	29 (42%)	50 (35%)	24 (34%)	3 (50%)
4	33 (15%)	12 (14%)	19 (18%)	2 (7%)	8 (19%)	15 (14%)	10 (14%)	20 (14%)	13 (19%)	0 (0%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
6	7 (4%)	3 (3%)	3 (3%)	1 (4%)	0 (0%)	6 (6%)	1 (2%)	3 (2%)	4 (5%)	0 (0%)
CPB time (min)	107 (67 - 135)	112 (67 - 149)	101 (64 - 134)	103 (80 - 124)	110 (85 - 131)	108 (63 - 132)	102 (70 - 144)	108 (68 - 140)	103 (67 - 132)	104 50 - 140
Aortic cross clamp time (min)	44 (16 - 73)	52 (20 - 79)	39 (14 - 70)	51 (15 - 74)	50 (35 - 78)	39 (19 - 71)	39 (0 - 77)	45 (15 - 77)	47 (20 - 72)	0 (0 - 56)
Circulatory arrest time (min)	0 (0 - 4)	0 (0 - 5)	0 (0 - 1)	0 (0 - 2)	0 (0)	0 (0 - 4)	0 (0 - 8)	0 (0 - 5)	0 (0)	0 (0)
Lowest operative temp \leq 18°C	37 (15%)	19 (20%)	15 (13%)	3 (10%)	10 (20%)	17 (15%)	10 (13%)	27 (18%)	10 (14%)	0 (0%)

Table 6.3 Clinical and surgical characteristics for the whole study group and divided for individual genotype. For categorical data, data is presented as number of patients (% of group). For continuous variables, data is presented as median \pm IQR. There was no statistically significant difference in demographics and operative details within any of the cytokine promoter genotypes, nor between a particular genotype and the overall study group.

6.4.4 Outcome for the whole study group

The median duration of ventilation was 2.75 days (range 0 hrs – 86 days), and the median length of stay in the intensive care unit was 3.9 days (16 hours – 86 days). In the majority of patients (62%, 136/219) the LOS was ≤ 5 days, with the remainder (38%, 83/219) requiring a median of 12.6 days (6–86 days).

During the Intensive Care stay, nine patients (4.1%) fulfilled the criteria for sepsis and a further three patients for SIRS (frequency of sepsis/SIRS = 5.5%). All septic/SIRS patients were long stay patients. There was an overall mortality rate of 3.7% (8/219). These figures are consistent with the overall incidence of complications within the cardiac intensive care unit at Great Ormond Street Hospital. Seven patients died during the hospital admission and one after transfer back to the local hospital. Of the patients that died post-operatively, only one patient died in the first 5 post-operative days from a non-infective cause. Two of the patients who died also fulfilled the criteria for sepsis.

6.4.5 Distribution of Genotyping

Genotyping for all promoter polymorphisms revealed a distribution that did not differ significantly from that predicted by the Hardy-Weinberg equilibrium law utilising predicted frequencies from other UK studies (Table 6.4). The population studied here was however, very mixed in ethnicity. A large number of the patients who have cardiac surgery in this centre are referred from overseas, particularly the Arab states. There does not appear to be any previously published studies describing the allele or gene frequencies of these promoter polymorphisms in an Arab population.

Patient characteristics and measures of surgical stress were comparable between the various haplotypes (Kruskal-Wallis $p>0.05$). This was true even for TNF- α -308A homozygotes who, whilst few in number, were noticeably older, had different surgical repairs undertaken, and required neither aortic cross-clamping nor circulatory arrest during their surgery.

Haplotype	Predicted frequency (%)	Observed frequency (%) in overall patient group (n=219)	Observed frequency in subgroup of study (n=42)
IL-6 -174 GG	36	39.7	43 (n=18)
GC	45.5	47.5	36 (n=15)
CC	18.5	12.8	21 (n=9)
IL-10 GCC/GCC	21.3	20.5	24 (n=10)
GCC/-	41.2	47.6	43 (n=18)
- / -	37.5	31.9	33 (n=14)
TNF- α GG	57-84	64.7	62 (n=26)
GA	16-40	32.6	38 (n=16)
AA	0-4	2.8	(n=0)

Table 6.4 The frequency of investigated genotypes in the study population did not differ significantly from the reported incidence in control populations.

TNF- α values are given as a range due to the combination of several study results(Allen, 1999 ;Brull, Leeson and others, 2002 ;Brull, Montgomery and others, 2001 ;Fishman, Faulds and others, 1998 ;Morse, Olomolaiye and others, 1999 ;Turner, Williams and others, 1997).

6.4.6 IL-6 genotype and inflammatory response to CPB

42 patients (subgroup within the study) had plasma cytokine levels measured. All patients had low levels of circulating IL-6 at surgical induction. Patients who were homozygous for IL-6 -174 CC had significantly lower IL-6 response during cardiac surgery (Kruskal-Wallis $p < 0.05$), and significantly lower systemic levels over the first 24 postoperative hours (Fig 6.4). This difference in circulating levels of IL-6 between the three genotypes persisted at 48 hours.

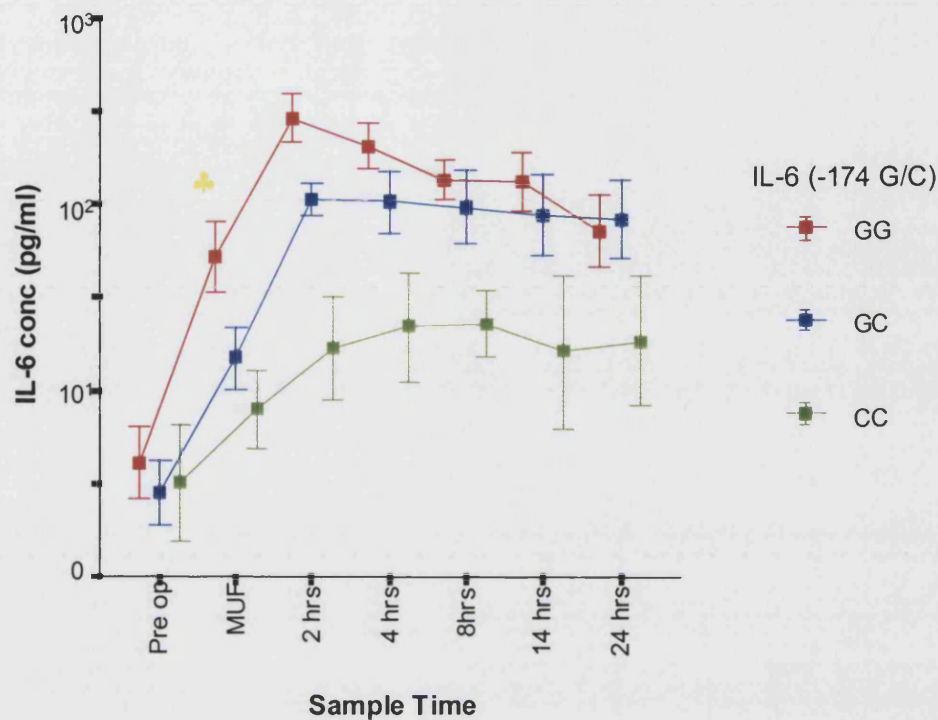


Figure 6.4 Patients who were homozygous for the IL-6 (-174) C/C haplotype had significantly lower systemic IL-6 levels following cardiac surgery involving cardiopulmonary bypass. All patients had blood collected for genotyping pre-operatively. Plasma was collected from induction of anaesthesia (pre op), at the end of surgery (MUF), through to 48 hours post-op, and levels of IL-6 were measured using ELISA. Patients were divided into 3 groups based on genotyping of the IL-6 -174 promoter polymorphism. Those patients that were homozygous for the IL-6 promoter polymorphism -174CC had significantly less circulating IL-6 at the end of surgery (* Kruskal-Wallis $p < 0.05$) and over the first 48 post-operative hours (ANOVA with Bonferroni correction $p < 0.001$).

There was no significant difference in the timing of peak IL-6 levels amongst the 3 genotypes, but there was a stepwise decrease in peak concentration of IL-6 from patients homozygous for the GG allele to those patients homozygous for the CC allele (Fig 6.5)

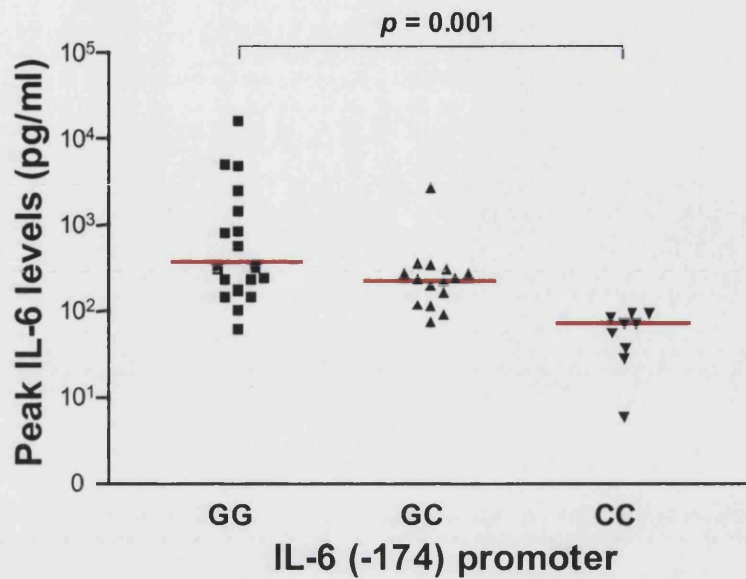


Figure 6.5 The presence of IL-6 -174CC promoter polymorphism significantly reduced the systemic peak of IL-6 seen following paediatric cardiac surgery.

There was a stepwise decrease in the peak concentration of IL-6 measured systemically in those patients who carried a C allele at position -174 of the IL-6 promoter.

6.4.7 IL-6 genotype and outcome

Outcome measures were examined for the whole study group (n=219). Twenty-eight patients (13%) were homozygous for IL-6 -174C haplotype. These patients had a significantly shorter duration of ventilation in the post-operative period (Kruskal-Wallis $p < 0.05$). There was also a trend for the patients who were homozygous for the CC promoter polymorphism to have a shorter length of stay compared to the rest of the patients; however this did not reach significance (Fig 6.6). There was no sepsis / SIRS or deaths among the IL-6 -174 C homozygotes.

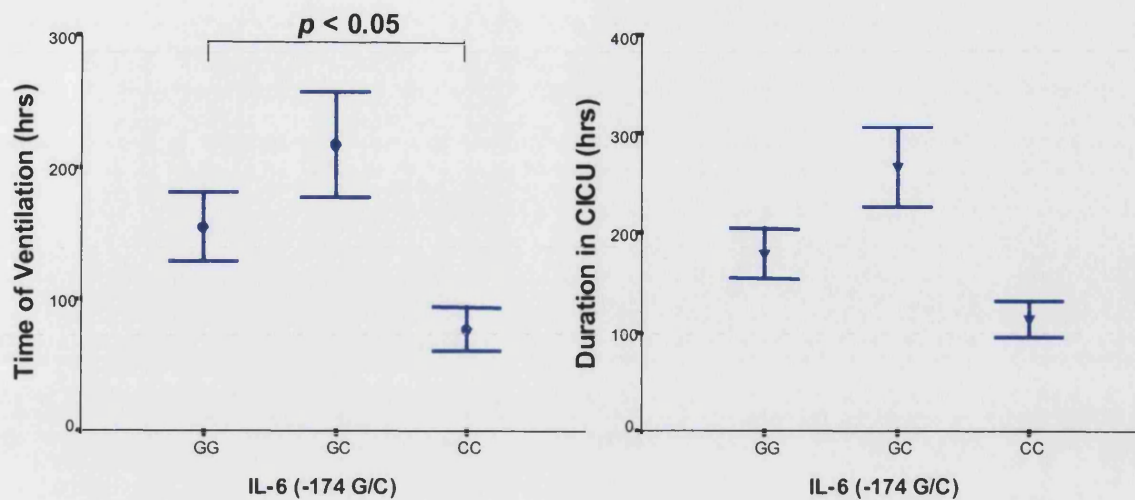


Figure 6.6 IL-6 genotype and outcome measures. Patients who were homozygous for the IL-6 -174CC haplotype had a significantly shorter duration of ventilation and trend toward shorter length of stay compared to the other haplotypes.

6.4.8 TNF- α genotype and inflammatory response to CPB

In the subgroup of patients in which plasma cytokines were measured, there were no homozygotes for the TNF- α -308A promoter polymorphism. There were 16 patients (34%) who were heterozygotes for the polymorphism. These patients had significantly lower levels of TNF- α systemically over the first 24 hours post-operatively (ANOVA $p = 0.002$) (Fig 6.7).

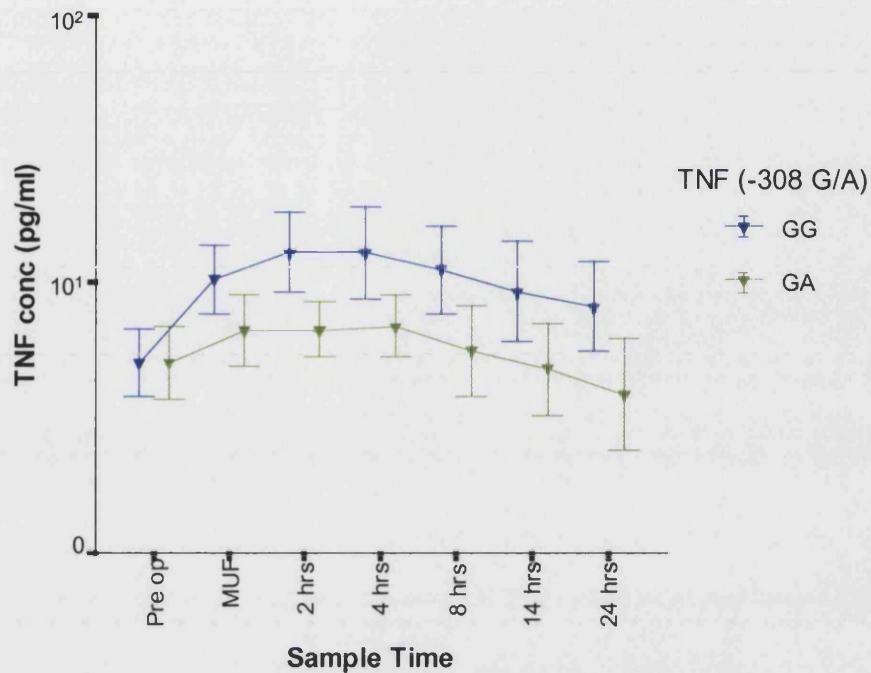


Figure 6.7 The presence of the TNF- α -308A allele was associated with significantly lower plasma levels of TNF- α post-operatively.

TNF- α levels following cardiopulmonary bypass were divided according to the presence or absence of the TNF- α -308 promoter polymorphism. The presence of the G/A substitution at -308 was associated with significantly lower levels of TNF- α over the first 24 hours post-operatively (ANOVA $p=0.002$).

6.4.9 TNF- α genotype and outcome

Six patients of the total study group (2.8%) were homozygous for the rare TNF- α -308A promoter polymorphism. Whilst few in number, these patients had significantly shorter ventilation requirements, and stayed less time in CICU compared to the overall study population (Fig 6.8). None of the patients homozygous for the -308A TNF- α promoter polymorphism developed sepsis/SIRS or died during the study. However whilst not significant, these patients tended to be older and had less technically difficult repairs with no periods of aortic cross clamping or hypothermia (Table 6.3).

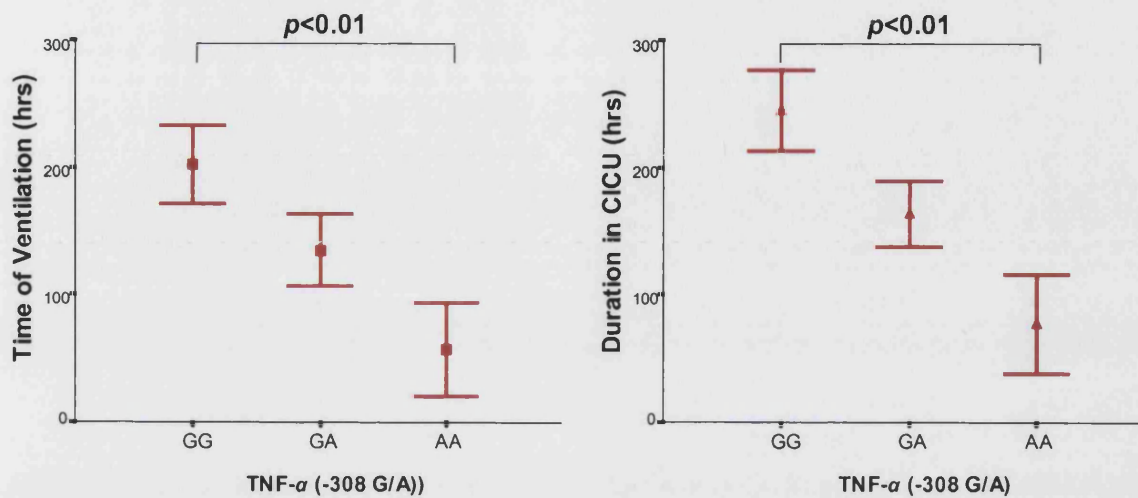


Figure 6.8 Those patients who were homozygous for the TNF- α -308A allele had significantly shorter duration of ventilation and length of stay in intensive care (Kuskal-Wallis $p < 0.01$).

6.4.10 IL-10 genotype, outcome and inflammatory response following CPB

Of the 42 patients in whom plasma IL-10 levels were measured 10 (24%) were homozygous for the GCC promoter polymorphism. These 10 patients had significantly greater systemic levels of IL-10 over the initial 24 post-operative hours (as determined by cytokine load) (Fig 6.9). There was a trend towards those patients who carried the GCC IL-10 promoter polymorphism to have reduced ventilation requirements and shorter duration of stay in the intensive care unit, but this did not reach significance (Fig 6.10).

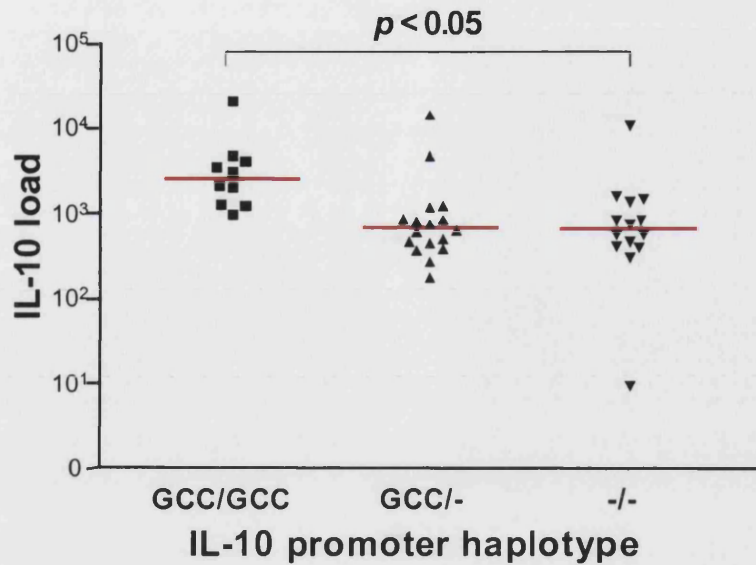


Figure 6.9 Circulating levels of IL-10 over the first 24 hours (IL-10 load) according to IL-10 promoter haplotype. Those patients who were homozygous for the GCC promoter polymorphism had significantly higher circulating levels of IL-10 in the immediate post-operative period ($p < 0.05$ Kruskal-Wallis).

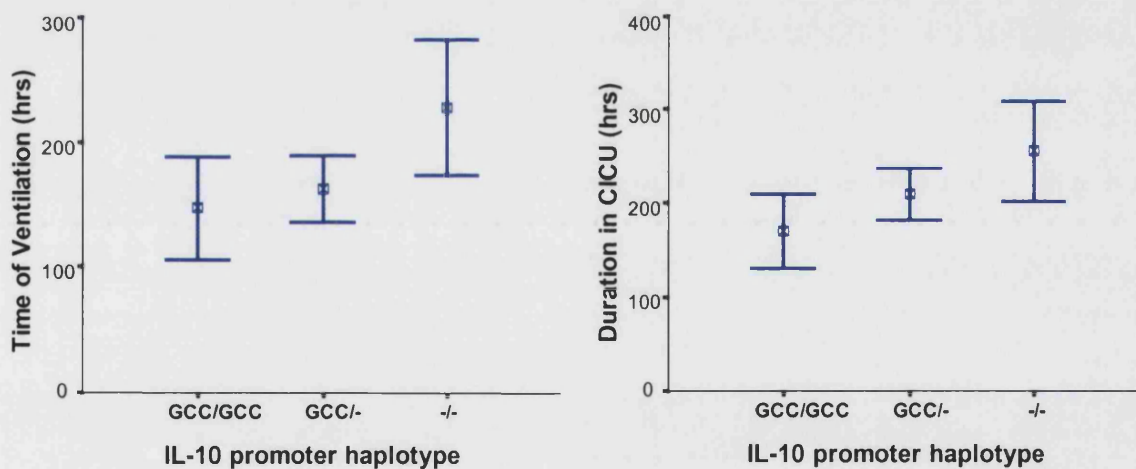


Figure 6.10 There was a trend toward reduced duration of ventilation and shorter intensive care stay in those patients who had the IL-10 GCC haplotype.

6.4.11 Effect of IL-10 genotype on systemic levels of pro-inflammatory cytokines

There was no direct discernable effect of IL-10 genotype on circulating levels of TNF- α or IL-6 in the immediate post-operative period.

6.5 DISCUSSION

Recovery from cardiac surgery is determined by many factors including the pre-operative physiological state of the patient, the success of the technical repair (always excellent if you ask the surgeons!), and the inflammatory response of the host to the insult.

This chapter describes a study designed to gain insight into the potential genetic regulation of the inflammatory response. Previous studies have shown that the IL-6, IL-10, and TNF- α genes all possess promoter polymorphisms which affect gene transcription and the production of the associated protein. High cytokine levels have been associated with the presence of the IL-6 and TNF- α alleles, and the IL-10 haplotype displayed in Table 6.3

(Allen, 1999 ;Burzotta, Iacoviello and others, 2001 ;Fishman, Faulds and others, 1998 ;Heesen, Kunz and others, 2003 ;Kroeger, Carville, and Abraham, 1997 ;Lowe, Galley and others, 2003 ;Stuber, Petersen and others, 1996 ;Tomasdottir, Hjartarson and others, 2003 ;Turner, Williams and others, 1997 ;Wilson, Gordon and others, 1994 ;Wilson, Symons and others, 1997).

Cytokine	Position	Allele
IL-6	-174	G
IL-10	-1082	G
	-819	C
	-592	C
TNF- α	-308	A

Table 6.5 Cytokine promoter polymorphisms associated with higher protein levels.

6.5.1 Association between cytokine polymorphisms and outcome

Results from 219 infants and children undergoing cardiac surgery involving CPB are presented here. The gene frequencies of the cytokine polymorphisms being investigated were in keeping with the frequencies previously published in other UK cohorts (Brull, Leeson and others, 2002 ;Brull, Montgomery and others, 2001 ;Fishman, Faulds and others, 1998 ;Morse, Olomolaiye and others, 1999 ;Turner, Williams and others, 1997). The presence of polymorphisms in the promoters of IL-6 and TNF- α had a significant association with duration of ventilation and/or length of stay in intensive care. There was also a trend for IL-10 promoter polymorphisms to be associated with outcome. Results presented here do not include HLA genotyping. This is the first description of these polymorphisms in paediatric surgical patients and to date there has been only one study in paediatric septic patients (Nadel, Newport and others, 1996).

In adults these 3 cytokine polymorphisms have been more extensively examined, however with frequently conflicting results. As in the results presented here, outcomes measures included duration of ventilation, length of stay, organ dysfunction and mortality. The most consistent finding has been with the TNF- α -308A allele. In adult studies this allele has been associated with adverse outcome in septic shock, cerebral malaria, and hepatitis B infection (Hohler, Kruger and others, 1998 ;McGuire, Hill and others, 1994 ;Mira, Cariou and others, 1999 ;Tang, Huang and others, 2000). Whilst the presence of the -308A allele has been shown to be over-represented in two studies of critically ill patients (Mira, Cariou and others, 1999 ;Reid, Perrey and others, 2002), it's association with susceptibility to, or outcome from sepsis (excluding septic shock) has not been supported by association studies (Emonts, Hazelzet and others, 2003 ;Reid, Perrey and others, 2002 ;Stuber, Udalova and others, 1995 ;Tang, Huang and others, 2000). In contrast, a single study of this promoter polymorphism in outcome following cardiac surgery, showed an association between the -308A allele and shorter times to extubation with lower risk of prolonged mechanical ventilation (Yende, Quasney and others, 2003).

No clear pattern has emerged in IL-6 and IL-10 promoter polymorphism outcome association studies to date.

6.5.2 Outcome and protein levels

As described in section 1.5.3, there are strong associations reported between some cytokine genotypes and associated protein levels in *in vitro* experiments. This makes it tempting to ascribe the outcome differences between genotypes to protein levels. However, when attempts have been made to assess genotype and protein levels with outcome, a clear association is frequently not observed (Gaudino, Andreotti and others, 2003 ;Lowe, Galley and others, 2003 ;Schluter, Raufhake and others, 2002).

In this study, findings supported an association between IL-6 -174G allele and the IL-10 GCC haplotype with higher circulating protein levels of these cytokines. This association would be predicted from studies performed *in vitro*. However in contrast to previously published *in vitro* and *in vivo* data, patients carrying the TNF- α -308A allele had lower circulating levels of TNF- α compared to the -308GG homozygotes (Abraham, French, and Dawkins, 1993 ;Bouma, Crusius and others, 1996 ;Heesen, Kunz and others, 2003 ;Jacob, Fronek and others, 1990 ;Louis, Franchimont and others, 1998 ;Stuber, Petersen and others, 1996 ;Wilson, Symons and others, 1997). Unfortunately there were no plasma levels of TNF- α available from -308AA homozygotes on which to confirm this finding.

6.5.3 Importance of circulating cytokine levels

Plasma cytokine levels measured in this and other studies are the cumulative response to the stress of surgery and bypass and come from many cellular sources. Circulating cytokine levels give no information about monocyte potential to respond to stimulation which would be a better correlate of the *in vitro* experiments suggesting an association between genotype and phenotype.

Associating genotype with plasma or serum levels of cytokines may be misleading as it fails to take into account local cytokine production and the complex regulatory mechanisms that control cytokine secretion. In addition intermittent sampling carries with it the risk of missing peak concentrations.

In the study reported here, patients who were homozygous for the rarer IL-6 -174 promoter polymorphism (CC), had significantly lower systemic levels of IL-6 post-operatively. The highest circulating levels of IL-6 were seen in those patients homozygous for the -174G allele. This association directly contrasts with the adult findings published by Brull (2001) and Jones (2001). The reason for this discrepancy is not clear. It may be a product of the sample timing. Results presented in this chapter looked at cytokine levels over 48 hours, examining both peak and load, whereas Brull and Jones analysed genotype against the cytokine level taken at a single time point, admitting in the earlier study that the relationship did not hold true if other time points were examined. Another possible reason for this difference may be the age of the study populations. IL-6's release occurs wherever there is an acute phase response. In older patient groups many other pathogenic processes may therefore contribute to circulating IL-6 levels under the stress of CPB – smoking, atherosclerosis, other end organ disease etc. Thus in adult studies, the IL-6 response is less likely to be solely due to the interaction between genetic potential and surgical stress, and more likely to be 'muddied' by confounders. In the results presented above, IL-6 levels probably reflect the stress of surgery with a only a small contribution from the underlying cardiac condition. This may explain why in the subset of patients in which genotype and phenotype were analysed such a clear relationship was seen.

6.5.4 Summary

The results presented in this chapter highlight the role that genetic predisposition may play in the host response to surgery and CPB. The presence of IL-6 -174C and TNF- α -308A promoter polymorphisms were associated with significantly shorter durations of ventilation and duration of intensive care support following surgery. It is important to point out that this is an association, rather than causation. Furthermore while it would be convenient to explain this association on the basis of cytokine levels, this is probably an over simplified view of the role of the promoter.

Despite the reservations expressed above, this and other studies have shown an association between outcome and genotype indicating a role for these genes in determining the host response to surgery. Future studies should concentrate on the

mechanisms of how these genes are operating in this clinical setting. In addition the value of this type of analysis should be viewed critically, particularly with regards to patient pre-operative risk stratification.

CHAPTER 7

Regulation of MHC Class II expression in Cardiopulmonary Bypass

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7.1 INTRODUCTION

Consistently throughout this work, cardiopulmonary bypass has been associated with reduced MHC Class II expression on circulating monocytes. This reduction in expression occurred rapidly over the first 24 hours, was maximal within the first 72 post-operative hours, and was related in time to an observed hypo-responsiveness of whole blood to LPS. Reduced MHC Class II expression could be shown to correlate with an increased susceptibility to secondary infections.

The molecular mechanism for this reduction however is unclear. It is known that MHC Class II expression is tightly regulated, and transcription is under the control of the Class II transactivator (CIITA). In normal circulating monocytes, excessive amounts of MHC Class II are present intracellularly in the Class II compartment (MIIC) awaiting peptide loading (Reith and Mach, 2001;Ting and Trowsdale, 2002). Recycling of the surface MHC Class II molecules occurs by re-endocytosis into the MIIC followed by re-expression with a new antigenic peptide (Cresswell, 1994;Pathak and Blum, 2000;Pinet and Long, 1998;Roche, Teletski and others, 1993). The reduction in monocytic MHC Class II expression in an adult cohort of septic patients has been shown to be a posttranslational effect, with no significant change in the rate of transcription of HLA-DR or CIITA (Fumeaux and Pugin, 2002). To date, this is the only clinical study examining the molecular mechanisms for reduced MHC Class II expression. IL-10 and TGF- β have been proposed as the mediators of this process as *in vitro* studies show that these cytokines downregulate MHC Class II by inhibiting exocytosis and recycling (de Waal, Haanen and others, 1991;Koppelman, Neefjes and others, 1997). However, in the clinical study mentioned above, the authors were unable to show a significant correlation between plasma levels of IL-10 and monocyte levels of HLA-DR expression or levels of MHC Class II-related gene expression.

Whilst no direct correlation has been shown, elevated plasma levels of IL-10 appear to be persistent finding in patients with monocyte deactivation and septic shock. Cardiopulmonary bypass causes an early systemic release of IL-10 in paediatric patients over a time course similar to monocyte MHC Class II expression reduction (Fig 5.7). In addition to inhibiting exocytosis and recycling, IL-10 has also been shown to

downregulate the transcription of CIITA and thus MHC Class II *in vitro* (Harton and Ting, 2000), raising a second possible site of action.

The aims of this chapter were:

- (1) To define which step(s) of the MHC Class II pathway was(were) affected by cardiac surgery involving cardiopulmonary bypass
- (2) To relate circulating IL-10 levels to changes in gene transcription as measured by mRNA levels

7.2 METHODS

Throughout this thesis an ongoing challenge has been the limited blood volume available. As the age of surgical repair decreases, so to does the patient's weight and the total volume of blood that is acceptable to request for study purposes. In order to gain meaningful information about where CPB was having its affect on MHC Class II surface expression, each patient required a series of sampling points pre- and post-operation. In addition blood obtained at each time point needed to be analysed for all steps of the MHC Class II pathway being investigated.

Most of the methods utilised in this chapter have been described in detail in Chapter 2. Additional methods are described below.

7.2.1 Measurement of plasma cytokines.

The ELISA technique used to measure plasma cytokines in chapters 4 and 5 was not appropriate to use in this patient group because of the sample volumes available. A commercially available multiplexed bead assay technique (Cytometric Bead Array) was used that is highly sensitive on small sample volumes. This assay uses a sandwich technique to capture and detect soluble cytokines. Capture antibody for each cytokine is attached to beads of a distinct size so that simultaneous assaying of cytokines in a single sample is possible. Bound cytokine is detected using a fluorescently labelled antibody

and quantitated by comparing the median fluorescent intensity of the detection antibody for a specific cluster of beads to a known standard.

Specifically, a 25µl solution of mixed human inflammation capture beads for IL-6, IL-8, IL-10, and TNF- α (10µl/cytokine/sample) was incubated with 50µl of patient plasma or standard for 1.5 hours. Samples were then washed and incubated for a further 1.5 hours with a detection antibody labelled with a PE fluorochrome. All incubations occurred in the dark. The samples were washed again and analysed on flow cytometry using BD FACSCComp Software (BD Biosciences, UK) (Fig 7.1).

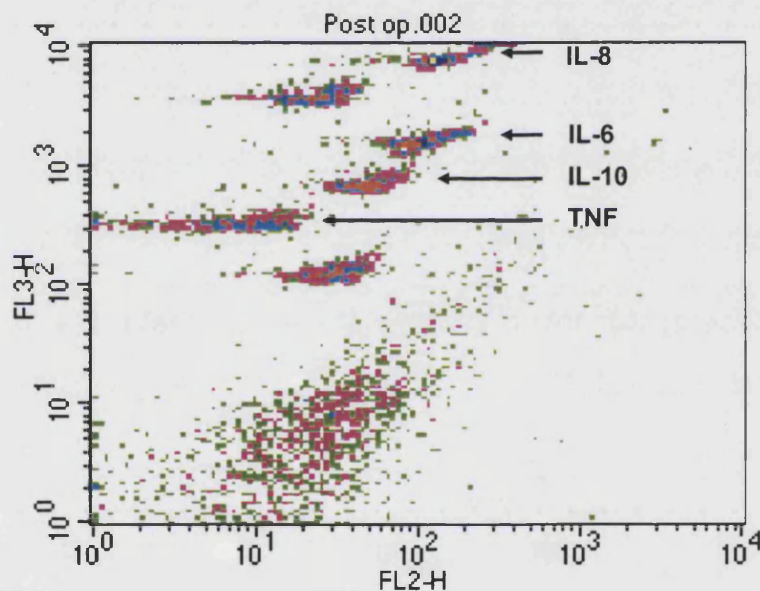


Figure 7.1 Appearance of plasma cytokine data using the multiplexed bead assay. Each assay contains clusters of capture beads with varying fluorescence (FL3-H) for the cytokines of interest. It was possible to measure 6 cytokines within this bead set (FL3-H 10^2 to 10^4). With increased levels of cytokine binding there is increased fluorescence from the detection antibody (FL2-H), moving the cloud generated to across to the right. The cytokines of interest were IL-8 (top cloud), IL-6 (3rd from the top), IL-10 (4th from the top), TNF- α (2nd bottom).

7.2.2 Semi-Quantitative Assessment of mRNA Levels

Real time reverse transcription-polymerase chain reaction was chosen as the most sensitive technique for mRNA detection and quantitation in view of the small sample volumes. This method was used to quantitate changes in post-operative gene expression for MHC Class II related genes compared to pre-operative expression.

7.2.2.1 Extraction of mRNA and generation of cDNA

A known number of monocytes isolated from 2mls of whole blood by Ficoll hypaque gradient and CD14 positive selection (Section 2.4.1) were resuspended in 200 μ l of cell lysis/binding buffer (to release message RNA) and frozen at -70°C to minimise degradation of mRNA, until all samples for that patient were obtained. This ensured that for each patient, serial samples were handled together and processed for quantification of mRNA under identical conditions.

Cell lysate was combined with 250 μ g of Oligo (dT)₂₅ covalently coupled to magnetic Dynabeads. The solution was incubated at room temperature with gentle tilting and rotation for 5 minutes during which the monocyte mRNA hybridised to the Oligo (dT)₂₅ sequence. The vial was placed back on the magnet, supernatant was removed, and the magnetic bead-mRNA complex was washed twice in Washing Buffer A before solution was changed to Washing Buffer B (Overview shown in Fig 7.2).

Complementary DNA was generated from mRNA by reverse transcription. The Dynabeads-mRNA-Buffer complex was placed on the magnet and supernatant removed. The Dynabeads-mRNA complex was resuspended in 11 μ l of RNase free water before adding 4 μ l of 5x first strand buffer, 2 μ l of 0.1M DTT, and 2 μ l of 5mM dNTP's. Each sample was placed at 42°C for 2 minutes, before 1 μ l of Superscript was added. Reverse transcription occurred at 42°C over 50 minutes, before the sample was placed at 70°C to heat-inactivate the transcriptase.

All patient samples were left undiluted in a total volume of 20 μ l. A normal control cDNA was prepared from 0.2 x 10⁶ monocytes by an identical method. Control cDNA was diluted to 1:3 with RNase free water and frozen in small aliquots at -20°C (final concentration mRNA from 0.33 x 10⁴ cells/ μ l).

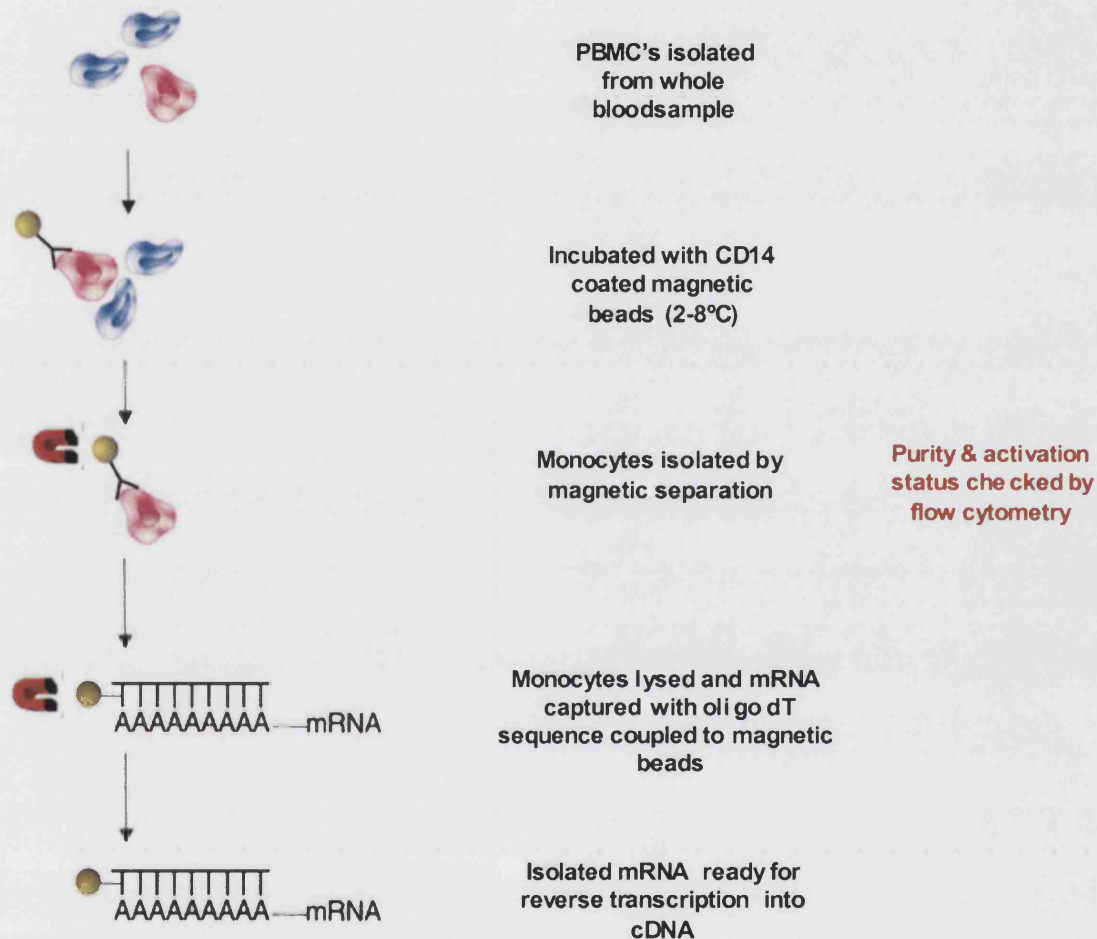


Figure 7.2 Schematic diagram of monocyte mRNA isolation from PBMC's.

Monocytes were positively selected from PBMC's before being lysed to release mRNA. Capture of mRNA was maximised by the use of an oligo-dT sequence coupled to magnetic beads. Isolated mRNA was converted to cDNA by reverse transcription, and stored for RT-PCR.

7.2.2.2 RT-PCR of MHC Class II related genes

Complimentary DNA for MHC Class II related genes were quantified using real time-polymerase chain reaction. The real time-PCR amplification of samples was performed using the TaqMan sequence detection system (ABI Prism 7000 SDS Software, Applied Biosystems, UK). Fluorescent probes and primers for MHC Class II and CIITA were obtained from the literature (Fumeaux and Pugin, 2002) and checked against the known gene sequence using Primer express (Applied Biosystem, UK). HLA-DRA was chosen as a constant sequence of genome that would allow quantitation of MHC Class II mRNA regardless of haplotype. Both probes were labelled with FAM and quenched with TAMRA. The details of the primers, probes and PCR mix are given in Tables 7.1 and 7.2.

	Oligonucleotide Sequence
CIITA Primer (Forward / Reverse)	5'-CCT GCT GTT CGG GAC CTA AA-3' 5'-GGA TCC GCA CCA GTT TGG-3'
CIITA Probe	5'-AGG GCC CAG CGC AAA CTC CAG T-3'
HLA-DRA Primer (Forward / Reverse)	5'-GCC AAC CTG GAA ATC ATG ACA-3' 5'-AGG GCT GTT CGT GAG CAC A-3'
HLA-DR Probe	5'-CAA CTA TAC TCC GAT CAC CAA TGT ACC TCC AGA G-3'

Table 7.1 Oligonucleotide sequences of the primers and probes used for RT-PCR

Real time PCR was performed in triplicate and all samples from a single patient were prepared and run together. Complementary DNA from a positive control was run with each RT-PCR.

PCR Mix	Volume	Final Concentration
Universal Master Mix	12.5 μ l	-
Forward Primer [5 μ M]	4.5 μ l	900nM
Reverse Primer [5 μ M]	4.5 μ l	900nM
Probe [5 μ M]	1 μ l	200nM
cDNA Template	2 μ l	-
Water	0.5 μ l	-
Reaction volume	25 μ l	

Table 7.2 PCR Mix for RT-PCR.

Volumes and where appropriate, final concentrations for reagents used per PCR reaction.

RT-PCR quantitates the amount of reaction product for each sample in every PCR cycle, by assessing the increase in fluorescence (OD). To quantify the results, a comparative threshold (C_t) method was utilised. The threshold is set at a point at which all the reaction rates have ceased to be exponential and entered a linear phase of amplification. The C_t is the fractional cycle number at which the amplified cDNA reaches this fixed threshold.

The number of monocytes in each sample from which the mRNA was collected, was divided by the mean C_t for the 3 replicas and multiplied by 10 to give a value of expression.

7.3 RESULTS

7.3.1 Optimisation of techniques to be used

A number of techniques to be used in this chapter needed to be optimised first in adult controls to ensure sensitivity when small blood volumes were utilised.

7.3.1.1 Isolation of monocytes

7.3.1.1.1 *Purity*

Monocytes were purified from whole blood using density gradients and positive selection as described in Chapter 2, section 2.4.1. Decreasing initial volumes of whole blood resulted in a loss in monocyte purity when a second density gradient was used (Fig 7.3). Attempts at isolating monocytes from 5ml or less resulted in only 70-80% of isolated cells being CD14 positive. In contrast positive selection using CD14⁺ beads and MACS column persistently returned a >95% purity even when as little as 2-3ml of whole blood was used.

7.3.1.1.2 *Monocyte activation*

For small whole blood sample volumes CD14⁺ positive selection yielded the greatest purity. The degree of cellular activation as a result of isolation was examined at both the density gradient and positive selection steps. There was minimal change in MHC class II surface expression following separation of PBMC's. However positive selection using MACs columns resulted in increased mfi (Fig 7.4). For this reason in subsequent work, expression of monocyte surface and intracellular MHC Class II was assessed after separation of PBMC's.

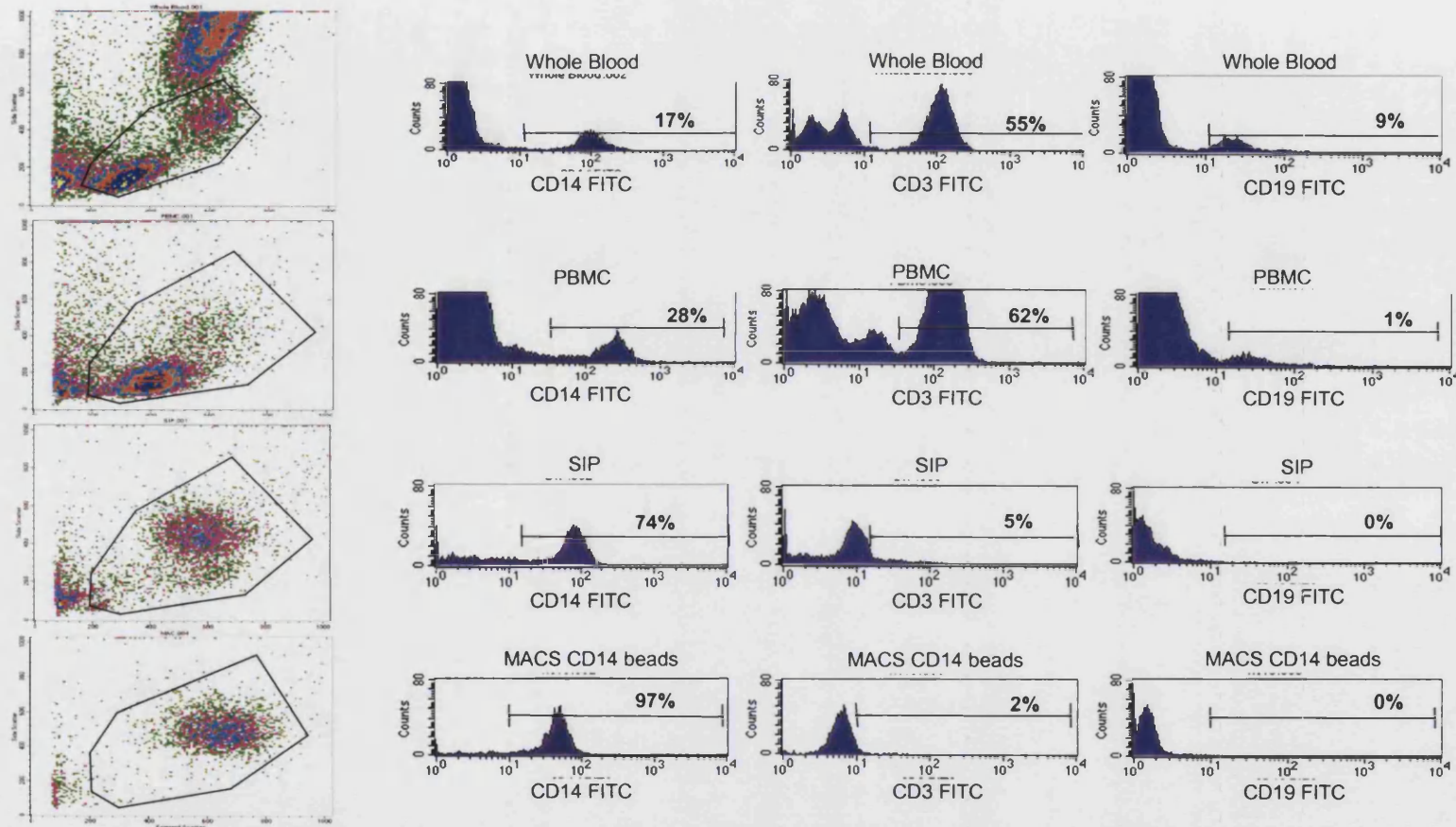


Figure 7.3 Percentage of monocytes, T cells and B cells are compared in whole blood, following PBMC isolation, SIP gradient and CD14 positive selection. Three millilitres of whole blood was collected and 50 μ l stained with monoclonal antibodies to CD14 (marker of monocytes), CD3 (marker of T-cells), and CD19 (marker of B-cells). Percentage positive cells within the gated area on the forward scatter – side scatter plot is given above the marker. The whole blood sample was then placed on a Ficoll-gradient and PBMC's isolated. PBMC's were divided and further purified using either a SIP gradient or CD14 positive bead selection. At each stage, cells were stained with the CD14, CD3, and CD19 monoclonal antibodies, and percentage purification calculated.

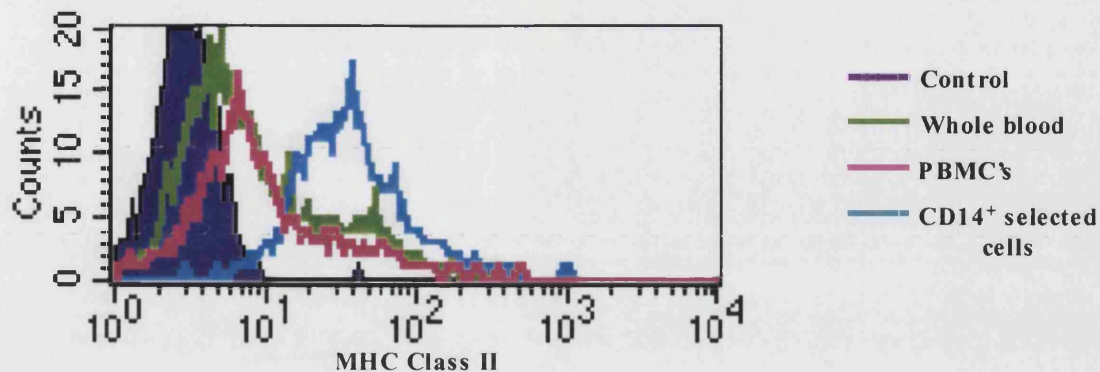


Figure 7.4 MHC Class II expression on the surface of patient monocytes (% and mfi) was increased by the purification processes. At the time points where MHC Class II expression was lowest on the surface of monocytes stained in whole blood, there was a marked increase in expression of this protein on monocytes separated by positive selection.

7.3.1.2 Optimisation of Confocal staining

Preliminary experiments to determine the most suitable fluorescent antibody for confocal imaging were performed on control blood. Comparisons in intensity of staining were made between the pan-MHC Class II antibody and a specific HLA-DR antibody, and between directly conjugated and double staining techniques. The most intense staining was obtained using a directly conjugated FITC labelled HLA-DR monoclonal antibody (Fig 7.5). There was no visible fluorescence in cells incubated with an isotype control monoclonal antibody (Fig 7.6).

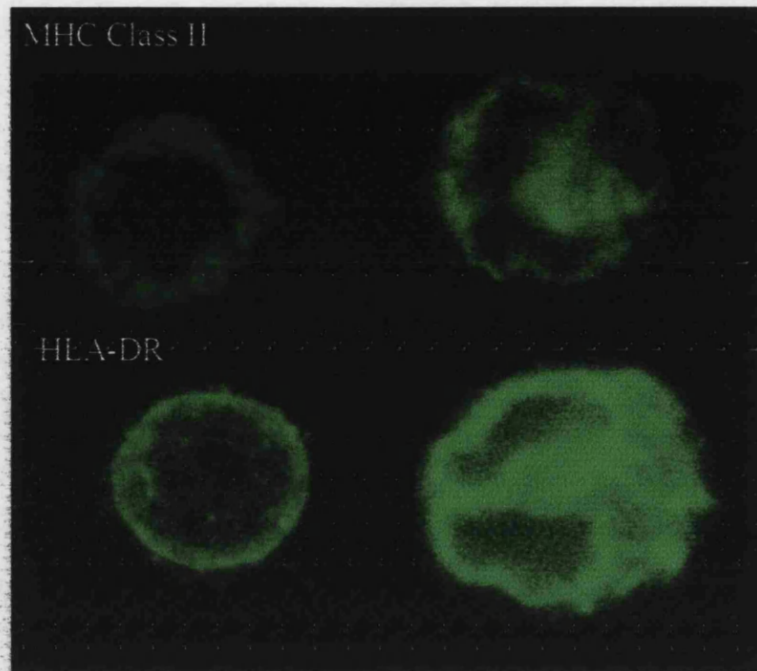


Figure 7.5 Comparison of FITC labelled monoclonal antibodies to MHC Class II and HLA-DR. Monocytes from normal adult controls with a high level of MHC Class II surface expression (>85%) were examined by confocal microscopy. Monocytes were identified by staining with a monoclonal antibody to CD14 (texas red). The directly conjugated monoclonal antibody specific to HLA-DR provided the best staining of both surface and intracellular MHC Class II at low laser voltages.

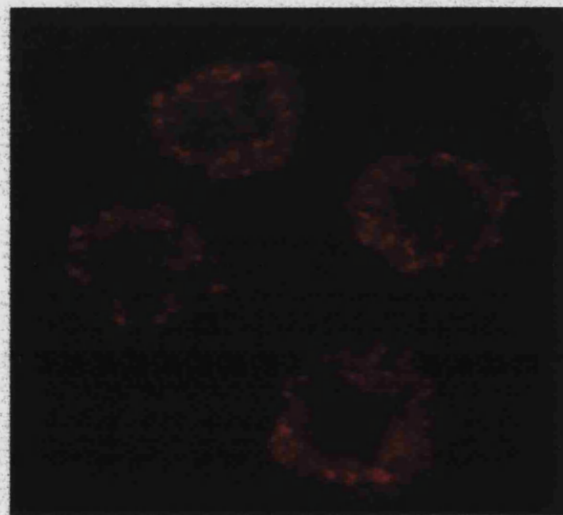


Figure 7.6 PBMC's surface stained with a second layer fluorochrome to CD14 (Texas red) and FITC labelled IgG1 isotype control (green). There is clear ring staining of the cell surface of monocytes with the texas red fluorochrome. In contrast there is no co-staining of the cell surface with the FITC fluorochrome.

A series of *in vitro* experiments were undertaken in adult control blood to ensure that a reduction in surface expression of MHC Class II on flow cytometry would be detectable by confocal microscopy. Culture of monocytes in the presence of IL-10 has been shown to reduce the surface expression of MHC Class II on monocytes (de Waal, Abrams and others, 1991; Koppelman, Neeffjes and others, 1997). In addition, culture of monocytes in the presence of LPS, TNF- α and IFN- γ has been shown to increase MHC Class II surface expression (Cella, Engering and others, 1997). Based on dose and time response curves (results not shown), PBMC's were cultured in the presence of IL-10 (10ng/ml), IFN- γ (100U/ml), and LPS (1ng/ml) at 37°C with 5% CO₂ for 24, 4, and 4 hours respectively (Fig 7.7). Following culture, cells were recovered and stained for both confocal and flow cytometry assessment of CD14, MHC Class II/HLA-DR surface expression.

Baseline MHC Class II expression (mfi) was 35-65 on flow cytometry. Co-culture with IFN- γ and LPS resulted a mild increase in MHC Class II expression (mfi: 47-112) which was not obvious on confocal microscopy. PBMC's cultured in the presence of IL-10 had a fall in surface expression of MHC Class II (mfi) which was detectable by both flow cytometry and confocal microscopy (Fig 7.8 and 7.9). This reduction in MHC Class II expression was partially restored by an additional 4 hours of culture with IFN- γ . This was seen using both flow cytometry and confocal microscopy

7.3.1.3 Summary

From these results, it was possible to develop protocols that would enable analysis of both surface and intracellular MHC Class II expression by both flow cytometry and confocal microscopy. Subsequent confocal microscopy and flow cytometry was performed on PBMC's separated by Ficoll hypaque gradients.

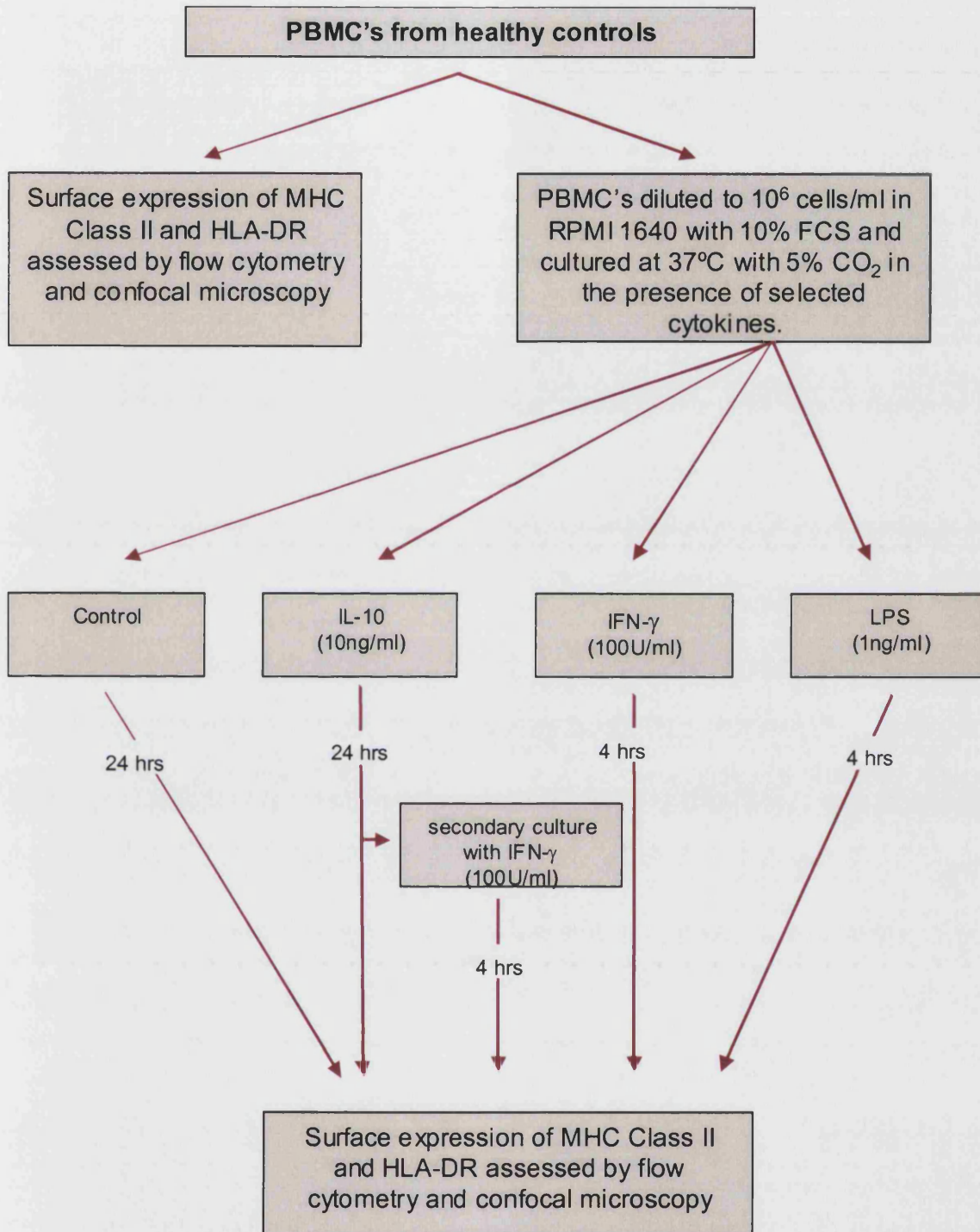


Figure 7.7 Optimisation experiments using PBMC's isolated from healthy adult controls. Peripheral blood mononuclear cells were isolated from 50ml of whole blood using a Ficoll hypaque gradient. Surface expression of MHC Class II and HLA-DR was examined using flow cytometry, and 10^5 cells were stained for confocal microscopy. The remaining cells were cultured for a variable amount of time with/without cytokines. Cells were washed twice and the cells from each culture plate were stained for CD14, MHC Class II and HLA-DR for both flow cytometry and confocal microscopy. A subgroup of cells cultured in the presence of IL-10 for 24 hours underwent secondary culture in the presence of IFN- γ before being stained.

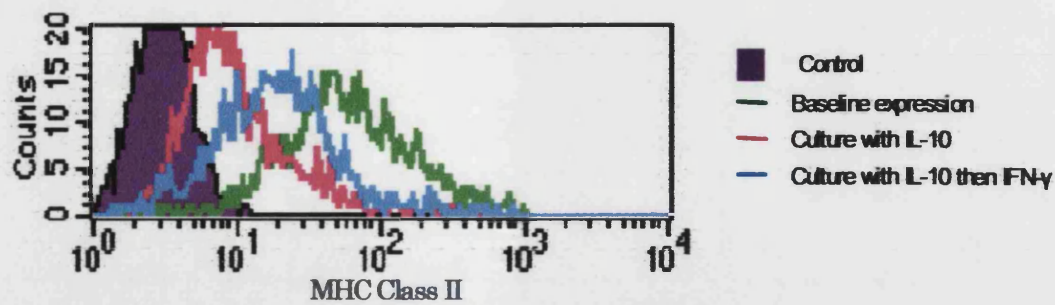


Figure 7.8 Effect of IL-10 on PBMC surface expression of MHC Class II. PBMC's were cultured with and without 10ng/ml of IL-10 for 24 hours. Cells were then assessed by flow cytometry for MHC Class II expression. A subgroup of cells was cultured for an additional 4 hours in the presence of 100U/ml of IFN- γ and surface expression of MHC Class II reassessed. Monocytes cultured in the presence of IL-10 showed a marked reduction in MHC Class II surface expression that was partially restored by an additional period of culture in the presence of IFN- γ .

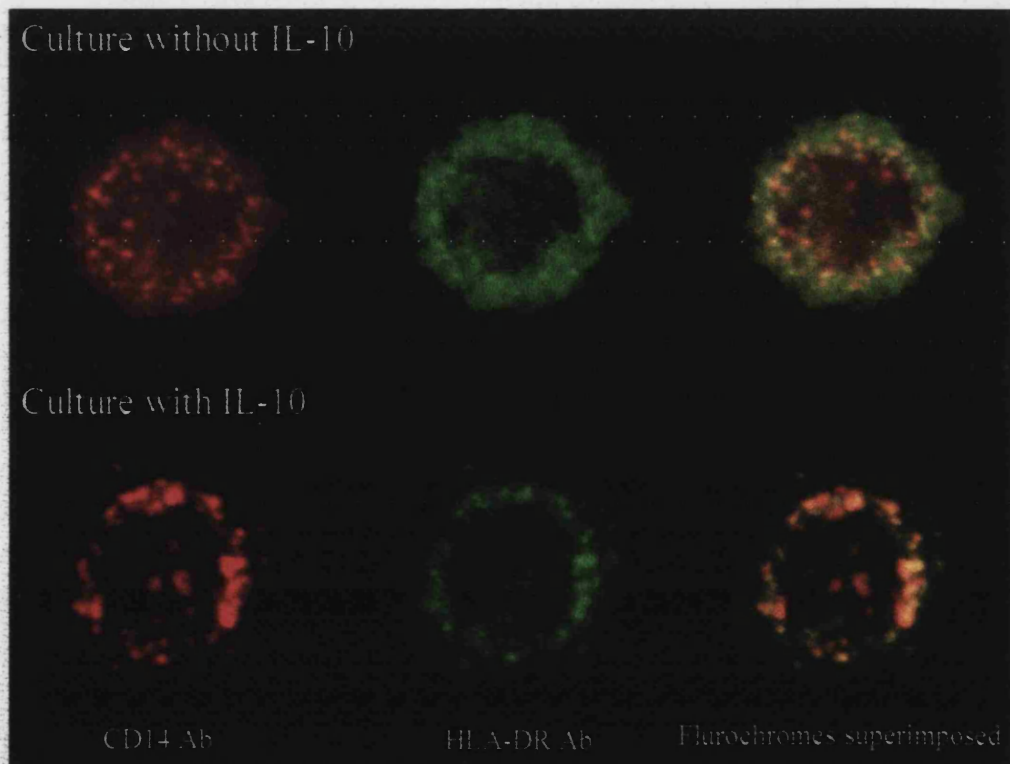


Figure 7.9 Effect of IL-10 on surface staining of HLA-DR. PBMC's were cultured in RPMI 1640 with and without IL-10 (100ng/ml) for 24 hours. Cells were washed and stained with fluorescently labelled monoclonal antibodies to CD14 (Texas red) and HLA-DR (FITC). The finding on flow cytometry of reduced surface expression of HLA-DR was easily confirmed with confocal microscopy.

7.3.2 Detection of MHC Class II localisation in Patients

Ten infants and children undergoing cardiac surgery involving CPB were randomly recruited over a 6 week period. Any child undergoing cardiac surgery involving CPB was eligible for this study. On each sample a series of experiments were performed in an effort to localise the primary cellular site at which the alteration in MHC Class II expression was originating.

An outline of the investigations performed at each time point is shown in Figure 7.10. Samples were collected pre-operatively, on completion of CPB (post op), and each morning for the first 3 post-operative days. Fifty microlitres of whole blood was stained for CD14 and MHC Class II expression. Plasma from one millilitre of blood was separated and stored at -70°C for cytokine analysis. PBMC's were extracted from the remainder 1-2ml of whole blood, and aliquots of 10^4 cells were stained for surface and intracellular assessment of MHC Class II expression by flow cytometry and HLA-DR expression by confocal microscopy.

Monocytes from the remaining PBMC's were isolated using CD14⁺ beads as described in the Chapter 2, section 2.4.1.2.2. A cell count was performed using a haemocytometer before the monocytes were lysed in 200µl of lysis/binding buffer and frozen at -70°C for RT-PCR of HLA-DRA and CIITA cDNA. The techniques of mRNA extraction, conversion to cDNA, and RT-PCR have been described in the methods above.

The patient characteristics followed by the results from each part of the study are presented below, with a final section summing up the results as a whole.

7.3.2.1 Patient characteristics, operative details and outcome

The demographic and operative details are presented in Table 7.3 and 7.4. Outcome measures included the patient's duration of ventilation and length of stay in the Intensive Care Unit. None of the 10 patients fulfilled the criteria for sepsis or SIRS. All patients survived to discharge.

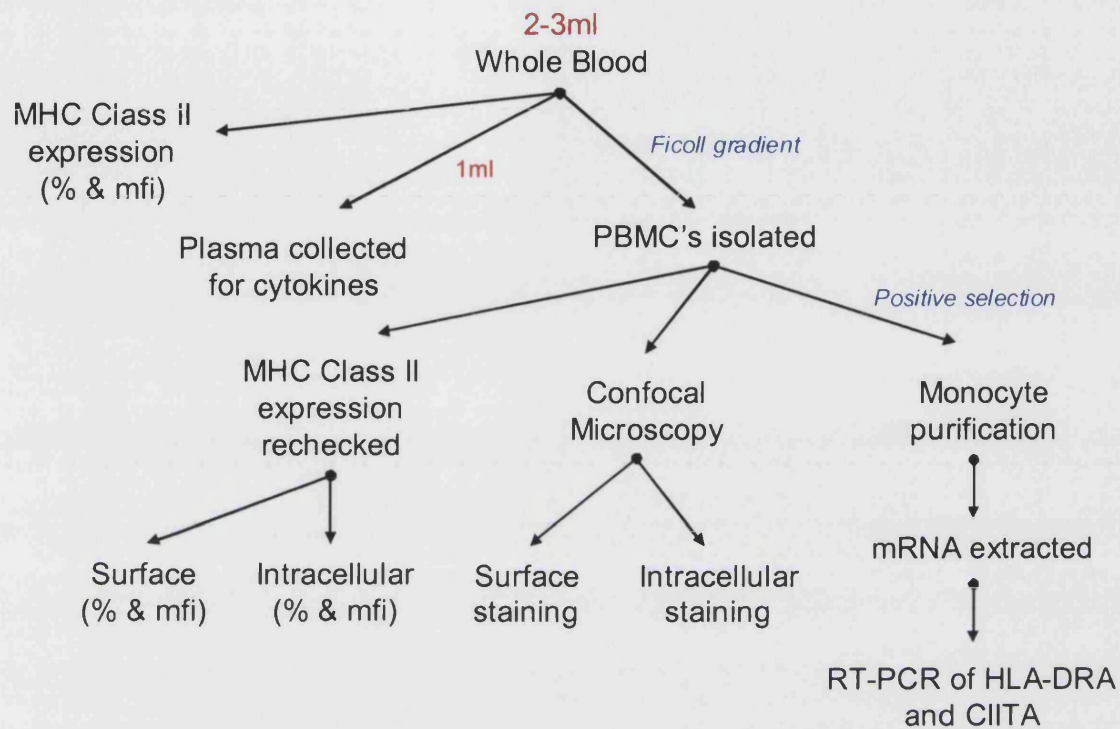


Figure 7.10 Study design. At each time point a number of investigations were performed in an effort to determine at what point MHC Class II expression is interrupted post-operatively. Surface expression of MHC Class II was examined on whole blood, before PBMC's were separated over a ficoll density gradient. PBMC's were examined for surface and intracellular MHC Class II staining by confocal and flow cytometry. After further purification monocytes were lysed for quantitation of message RNA levels.

Patient	Age (days)	Sex	Weight (kg)	Cardiac Lesion
1	222	F	5.60	Congenitally corrected transposition of great arteries, VSD, sub-pulmonary stenosis, severe tricuspid incompetence
2	20	F	2.72	Transposition of great arteries
3	1254	M	13.10	Double outlet right ventricle, VSD, Pulmonary outflow tract obstruction
4	122	M	4.72	VSD
5	449	M	9.60	Pulmonary atresia VSD, Major aorto-pulmonary collateral arteries
6	94	F	4.00	Coarctation of the aorta / Transverse arch hypoplasia VSD x2, Small left heart
7	99	F	3.80	Atrioventricular septal defect Patent ductus arteriosus
8	806	F	9.40	Tetralogy of Fallots (severe) Patent ductus arteriosus
9	226	M	7.88	Tetralogy of Fallots Tracheal stenosis
10	28	M	3.77	Transposition of great arteries

Table 7.3 The demographic details and cardiac diagnosis for the 10 patients enrolled. Ventriculoseptal defect (VSD)

Table 7.4 (over page) Operative details and outcome measures for the 10 study patients. Details of the cardiac repair performed, lowest temperature achieved during theatre, cardiopulmonary bypass (CBP), aortic cross clamp (X-clamp), and circulatory arrest time is given for each patient. Outcome measures including duration of ventilation and length of stay in the cardiac intensive care unit are also included. Ventricular septal defect (VSD), Atrial septal defect (ASD), Patent ductus arteriosus (PDA), Total cavopulmonary circulation (TCPC), Patent foramen ovale (PFO), Atrioventricular septal defect (AVSD), Tetralogy of Fallot's (TOF)

Table 7.4 Operative Details and outcome measures for the 10 study patients.

Patient	Cardiac Operation	Lowest operating temperature	CPB time (min)	X-clamp time (min)	Circulatory arrest time (min)	Duration of ventilation (hrs)	Duration in CICU (hrs)
1	Unable to perform planned switch due to severe pulmonary stenosis, Tricuspid valve replacement, Pulmonary valvotomy, VSD closure	22°C	159	112	0	64	93
2	Arterial switch ASD closure, PDA ligation	18°C	121	81	7	39	91
3	TCPC / Fontan	32°C	50	0	0	5	47
4	VSD closure PFO closure	28°C	103	50	0	21	42
5	VSD patch, unifocalisation of right collateral vessels, right ventricle to pulmonary artery conduit, ASD closure	23°C	202	48	0	31	44
6	Closure of multiple VSD's Pulmonary artery banding	16°C	92	54	36	97	186
7	AVSD repair Suture of mitral cleft, PDA ligation	28°C	98	69	0	119	165
8	TOF repair Bovine pericardial patch, Enlargement of main pulmonary artery, creation of small ASD, PDA ligation	28°C	68	39	0	13	66
9	TOF repair Slide tracheoplasty, PDA ligation	30°C	126	34	0	148	168
10	Arterial switch ASD closure	22°C	128	77	7	299	474

7.3.2.2 Relationship between surface and intracellular MHC Class II expression by flow cytometry

Whole blood and PBMC's were stained for surface and intracellular expression of MHC Class II. Consistent with the findings presented in previous chapters, surface expression of MHC Class II on monocytes (% & mfi) fell in all patients following cardiopulmonary bypass. MHC Class II surface expression (% & mfi) from monocytes in whole blood and PBMC's was significantly reduced at the end of CPB from pre-operative levels (Wilcoxon signed rank test $p < 0.05$), and remained significantly lower over the first three post-operative days (%: $p < 0.05$, mfi: $p < 0.01$ on Day 1-2 for whole blood, and % & mfi: $p < 0.05$ for PBMC) (Fig 7.11 & 7.12).

The percentage of monocytes staining intracellularly for MHC Class II remained high (>85%) both pre and post-operatively. However, the median fluorescent intensity (mfi) of monocyte staining fell significantly following bypass (Day 1 $p < 0.005$ Wilcoxon signed rank test) (Fig 7.12).

Monocyte Class II Expression (%)

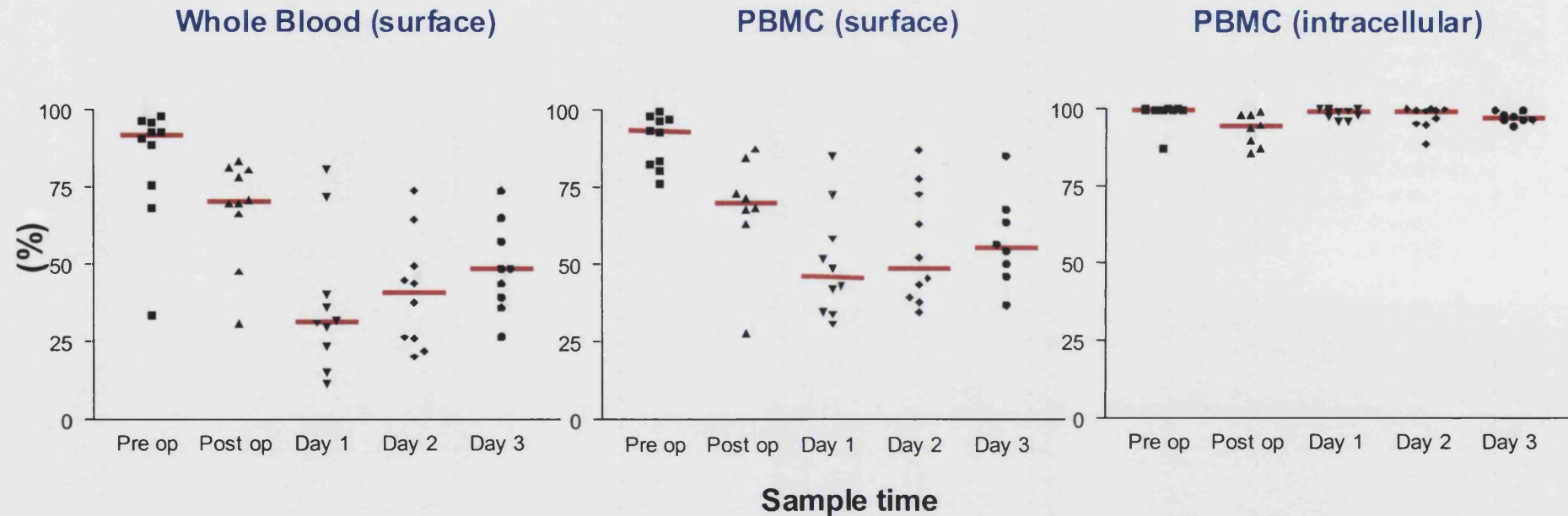


Figure 7.11 Changes in monocyte MHC Class II expression (%) with cardiac surgery involving CPB.

Ten patients had blood samples drawn pre and post-operatively for 3 days following their operation. Whole blood was dually stained with CD14 and MHC Class II fluorescently labelled monoclonal antibodies to CD14 and MHC Class II. PBMC's were separated from the blood and both surface and intracellular staining for MHC Class II was examined on CD14⁺ cells. There was a significant fall in surface expression of MHC Class II on CD14⁺ cells in whole blood which was mirrored in the paired PBMC sample. Intracellular staining for MHC Class II remained >85% post-operatively.

Monocyte Class II Expression (mfi)

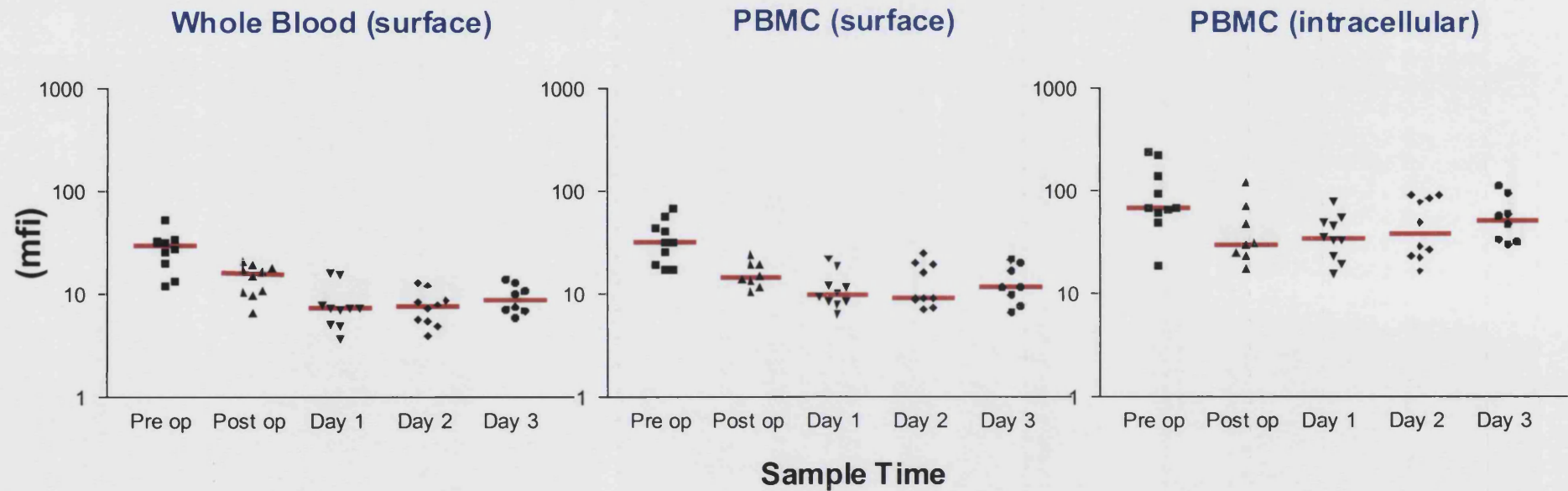


Figure 7.12 Changes in the median fluorescent intensity (mfi) of monocyte MHC Class II staining following CPB.

The intensity of intracellular staining for MHC Class II in circulating monocytes was a log fold higher than the corresponding surface staining. Cardiac surgery involving CPB resulted in a significant fall in the intensity of both a surface and intracellular MHC Class II staining (mfi) which was greatest on Day 1 and Day 2 ($p < 0.005$).

7.3.2.3 Monocyte HLA-DR distribution using confocal microscopy

Confocal microscopy was used to investigate the cellular localisation of the MHC Class II protein, and confirm the results obtained from flow cytometry. No attempts to quantitate fluorescence were made using the confocal. However the images analysed and the example shown below were representative of the cells seen on each slide. Figure 7.13 shows a series of fluorescent images from a monocyte stained for intracellular HLA-DR. Each image is 0.5µm apart. The top image is a computer generated composite of the series of images. All images subsequently shown in this chapter are single optical slices captured from the central part of the cell, corresponding approximately to the 2nd-3rd of the optical slices presented at the bottom of figure 7.13.

In addition to HLA-DR, all cells were stained with To-pro-3 iodine nuclear stain (Chapter 2 section 2.8.3), and monocytes were selected for imaging based on their cell morphology. When images were taken at 800x magnification, the To-pro-3 iodine nuclear staining was rapidly bleached, and recorded images presented here frequently appear absent for this fluorochrome.

7.3.2.3.1 *Surface HLA-DR expression*

In all 10 patients studied, monocytes had a noticeable reduction in surface HLA-DR fluorescence by confocal microscopy in the post-operative samples when compared to pre-operative expression. It would be expected that confocal findings would parallel the median fluorescent intensity (mfi) changes in monocyte MHC Class II surface expression measured by flow cytometry. The change in surface fluorescence by confocal microscopy was more dramatic than that suggested by the corresponding change in mfi on flow cytometry (Fig 7.14). For all 10 patients, confocal microscopy showed a reduction in surface HLA-DR expression immediately following bypass, which persisted over the first 24-48 hours. Five patients appeared by microscopy to have some recovery in surface fluorescence during the study period (patient 1,3,5, 6, & 10).

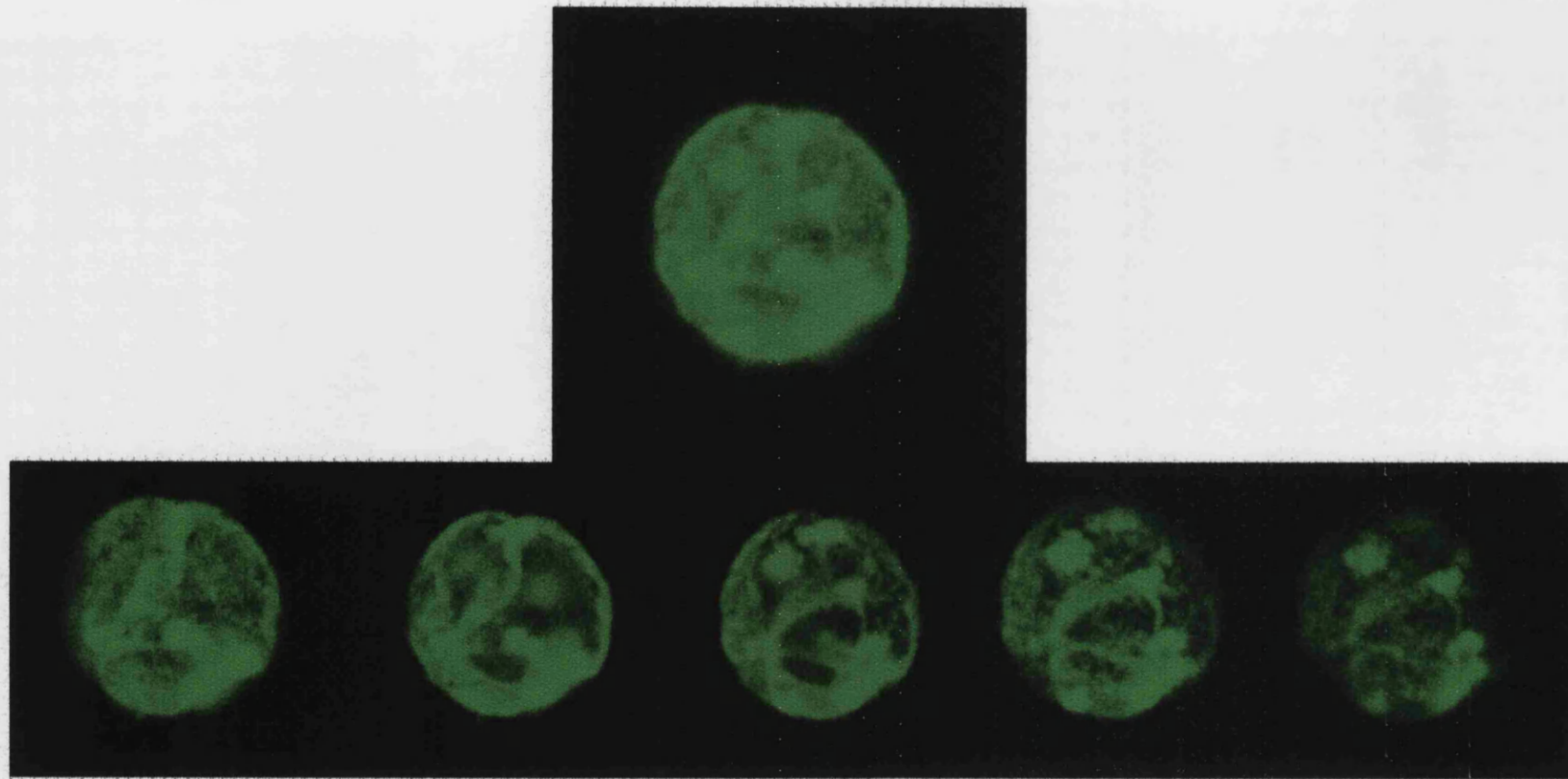


Figure 7.13 Images from confocal microscope of a monocyte with FITC staining for intracellular HLA-DR.

The bottom series of images show the intracellular distribution of HLA-DR in a single monocyte. Images are stored from a series of Z-sections (0.5 μ m apart), and can then be superimposed to form a single picture of cellular staining (top image).

Patient 5: MHC Class II by flowcytometry

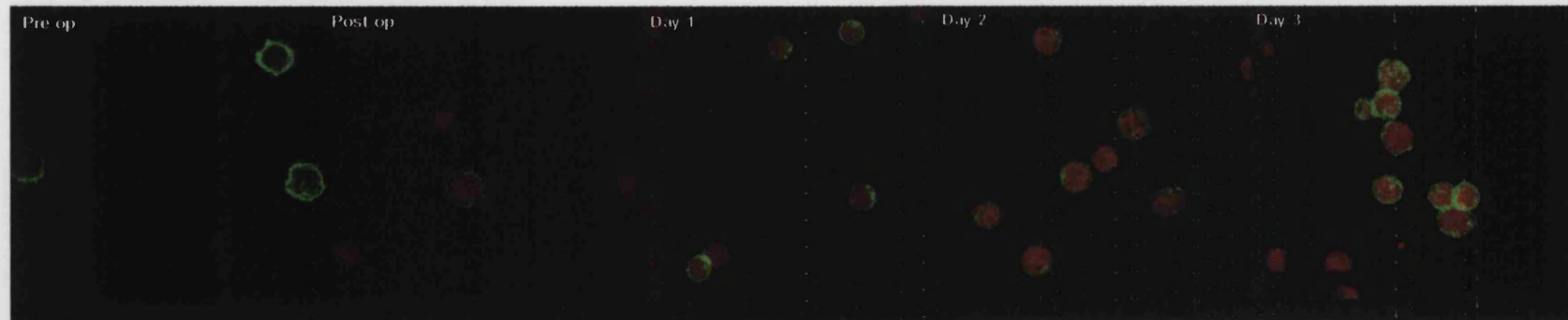
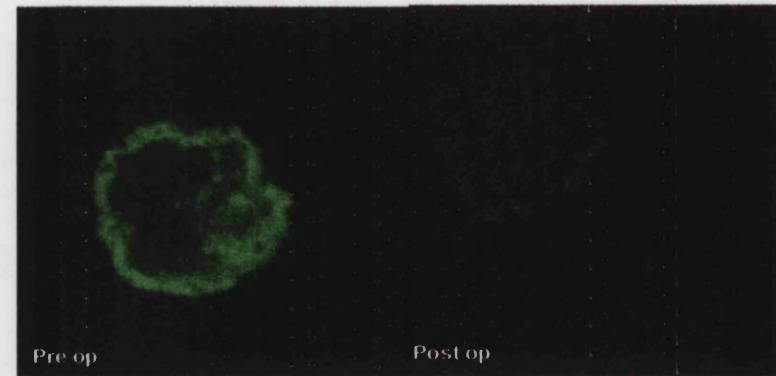
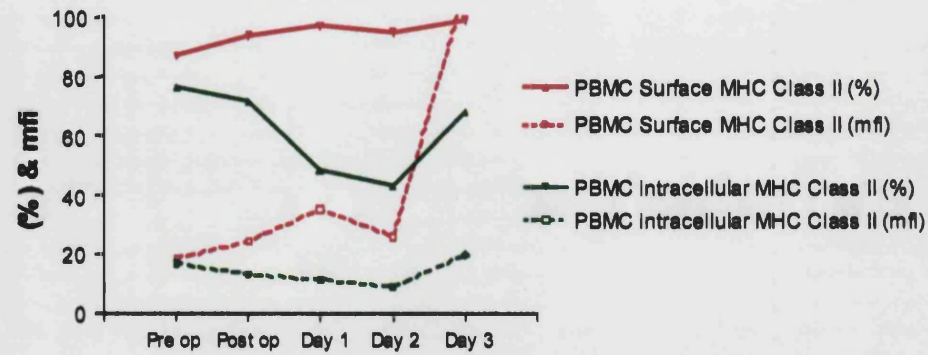


Figure 7.14 Surface fluorescence HLA-DR on monocytes, examined by confocal microscopy (Patient 5)

There was a reduction in HLA-DR surface fluorescence from pre-operative levels immediately post-op. HLA-DR fluorescence appeared to increase on circulating monocytes on Day 3. When compared to flow cytometry, confocal findings appeared to reflect changes seen in MHC Class II surface expression (% & mfi).

7.3.2.3.2 *Intracellular HLA-DR expression*

There was marked variation in the degree of intracellular HLA-DR staining seen on confocal microscopy amongst the pre-operative samples. Most patients had strong intracellular staining that decreased in the immediate post-operative period. A representative patient of this is illustrated in Figure 7.15. Intracellular staining of this patient's monocytes, showed reduction in HLA-DR fluorescence on the post-operative and Day 1 samples, but return of bright fluorescence on Day 2 and 3. One of the ten patients deviated from this pattern with low levels of HLA-DR pre-operatively, and increased fluorescence by Day 3 (Fig 7.16). These patterns were reflected by the median fluorescent intensity (mfi) of intracellular staining (inset top right corner of Figs 7.14-7.16).

Patient 7: MHC Class II by flowcytometry

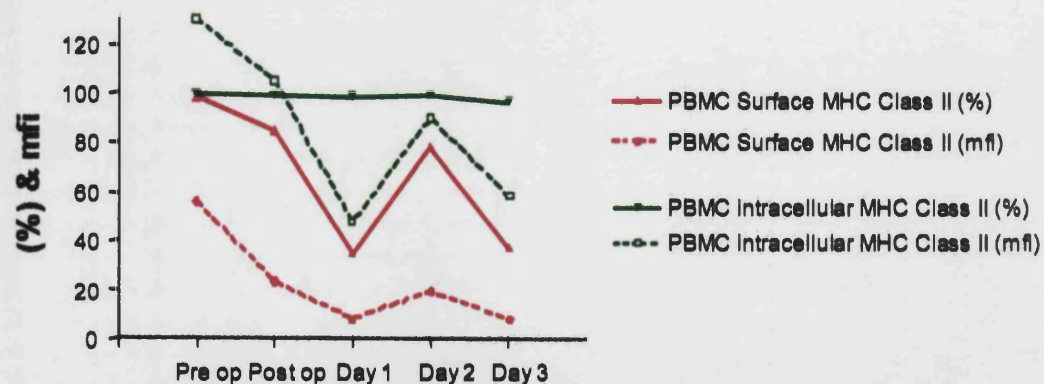
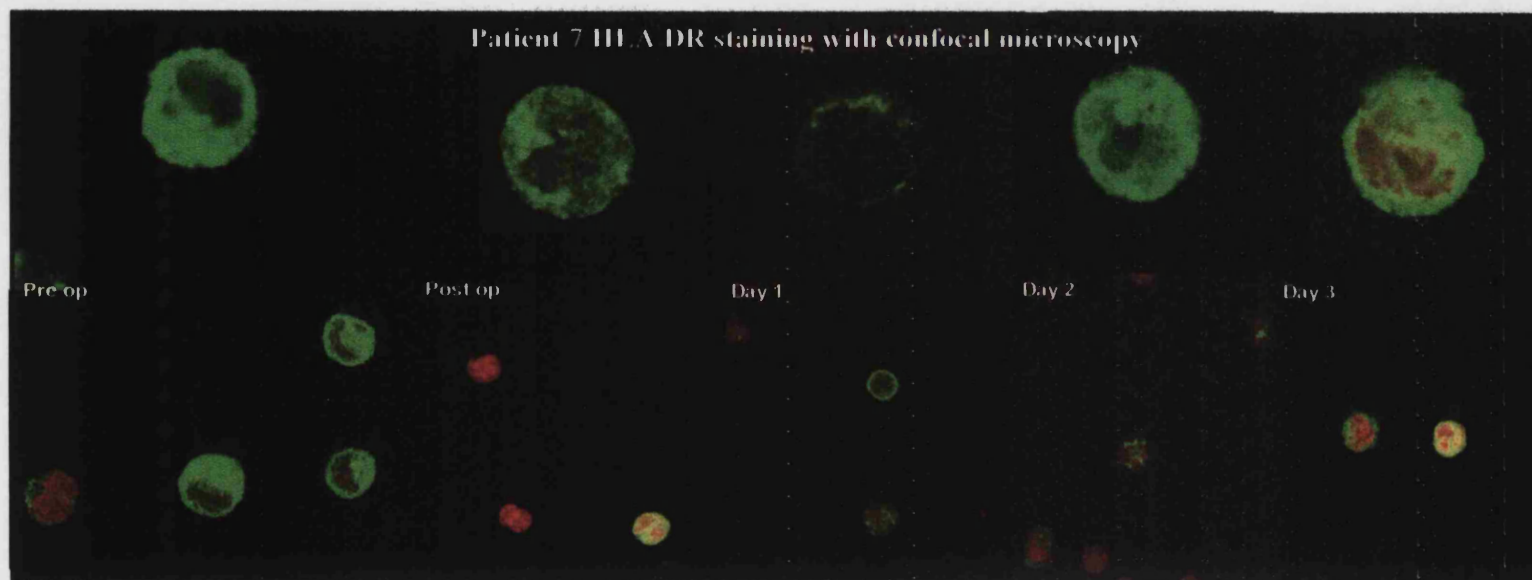


Figure 7.15 Intracellular staining of circulating monocytes for HLA-DR. Confocal microscopy was used to examine monocyte intracellular staining for HLA-DR and compared to results obtained by flow cytometry. Single mid optical sections of the cell are shown. Monocytes can be seen to be brightly fluorescent pre-operatively. Cells staining positive for just the nuclear stain were identified as lymphocytes by morphology. Staining appeared markedly reduced on the first post-operative day, but improved on Day 2 and 3. Confocal fluorescence appeared to reflect similar changes to those seen in the median fluorescent intensity (mfi) of MHC Class II staining assessed by flow cytometry



Patient 5: MHC Class II by flowcytometry

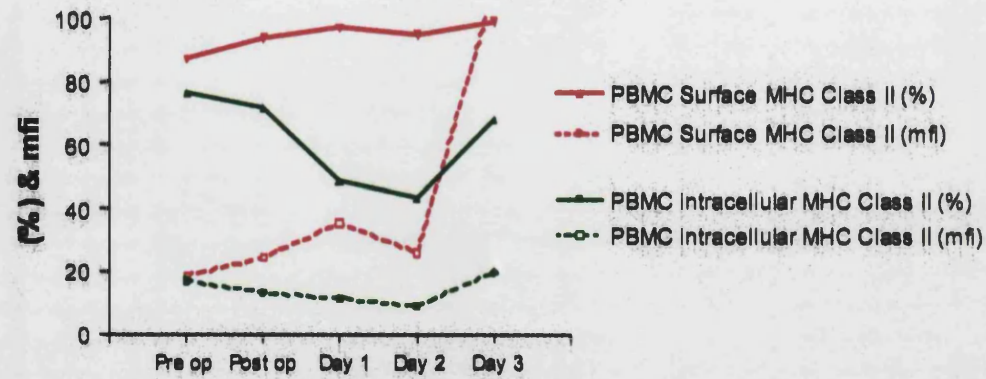
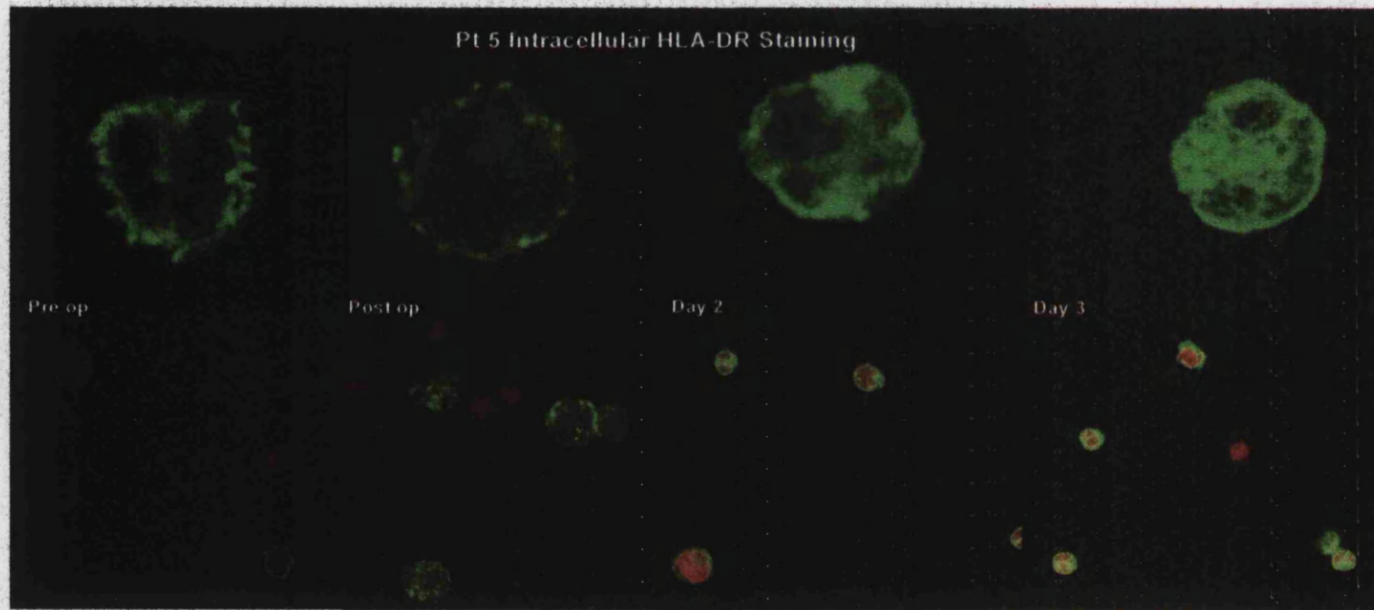


Figure 7.16 HLA-DR intracellular staining on confocal microscopy mirrors the median fluorescent staining of monocytes by flow cytometry. Patient 5 had low preoperative intracellular MHC Class II levels (mfi) on flow cytometry. Flow cytometry suggested that intracellular content of MHC Class II increased by the 3rd postoperative day. Examination of monocytes from the same blood samples by confocal microscopy confirmed these results.



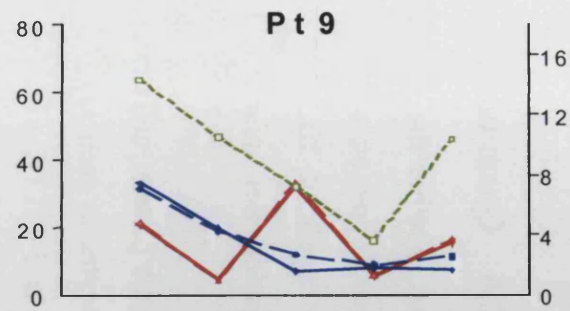
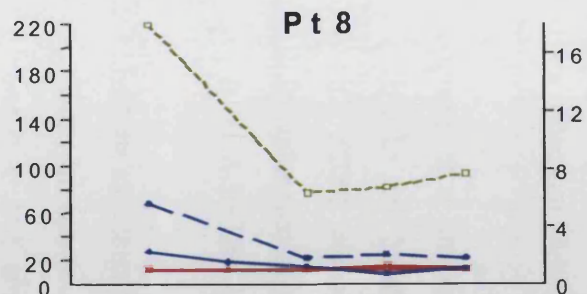
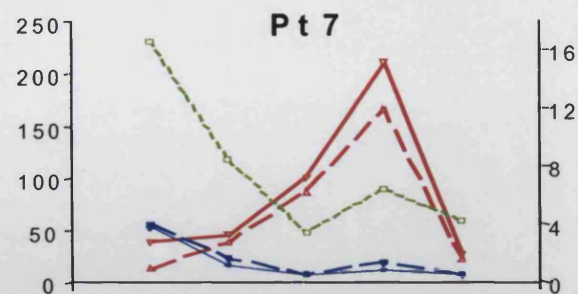
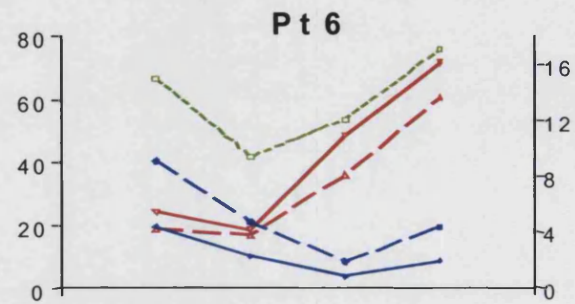
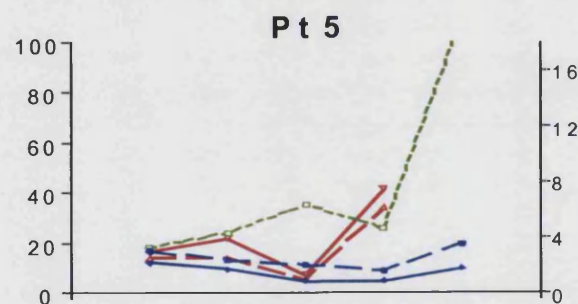
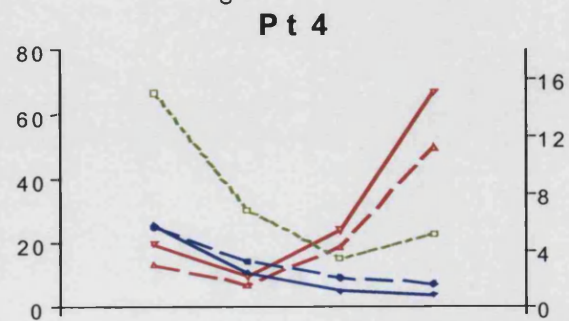
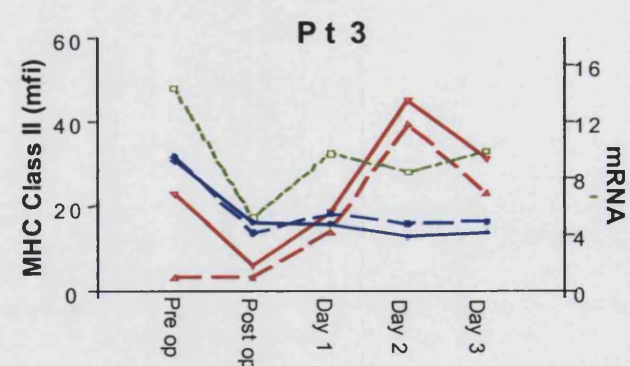
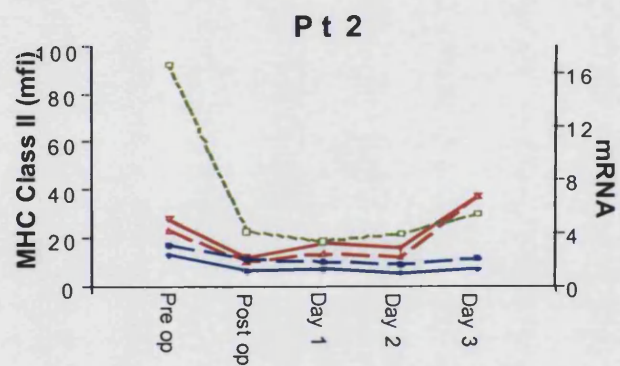
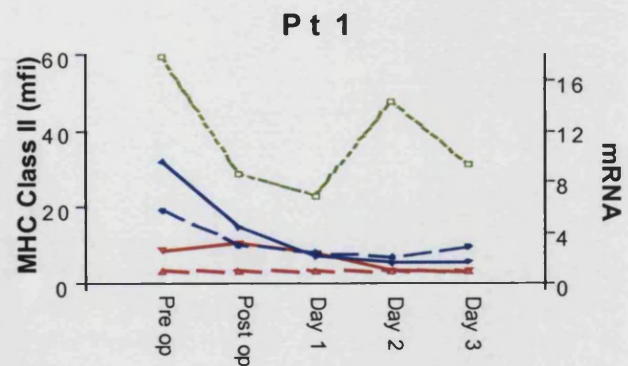
7.3.2.4 Quantitation of HLA-DRA and CIITA mRNA

To test whether the decrease in monocyte MHC Class II expression observed in patients following cardiac surgery was due to a transcriptional effect, messenger RNA levels of HLA-DR and CIITA, the molecule critical for MHC Class II expression, were quantified by real time-PCR.

For each patient, messenger RNA was obtained from a >95% purified population of monocytes at each sampling point. In all ten patients, levels of mRNA for HLA-DRA and CIITA were low prior to surgery (Fig 7.17). Gene transcription did not appear to increase at the end of the surgery. However by Day 2, seven of the 10 patients had increased levels of both CIITA and HLA-DRA mRNA. One additional patient had increased mRNA levels on day 3.

Consistent with previously published reports, levels of HLA-DRA expression paralleled that of CIITA expression (Fig 7.18)(Sims and Halloran, 1999;Ting and Trowsdale, 2002). The timing of the samples was too infrequent (24 hourly) to detect the reported delay (Sims and Halloran, 1999;Ting and Trowsdale, 2002) between CIITA and HLA-DRA gene expression.

All the patients studied in this group had a fall in monocyte MHC Class II expression (both % and mfi) that preceded the increased transcription in CIITA and HLA-DRA. Patients 3, 4, 5, 6 and 10 had an increase in intracellular MHC Class II expression within 24 hours of the increase in MHC Class II related mRNA. One additional patient (pt 9) had an increase 48 hours after a rise in mRNA levels.



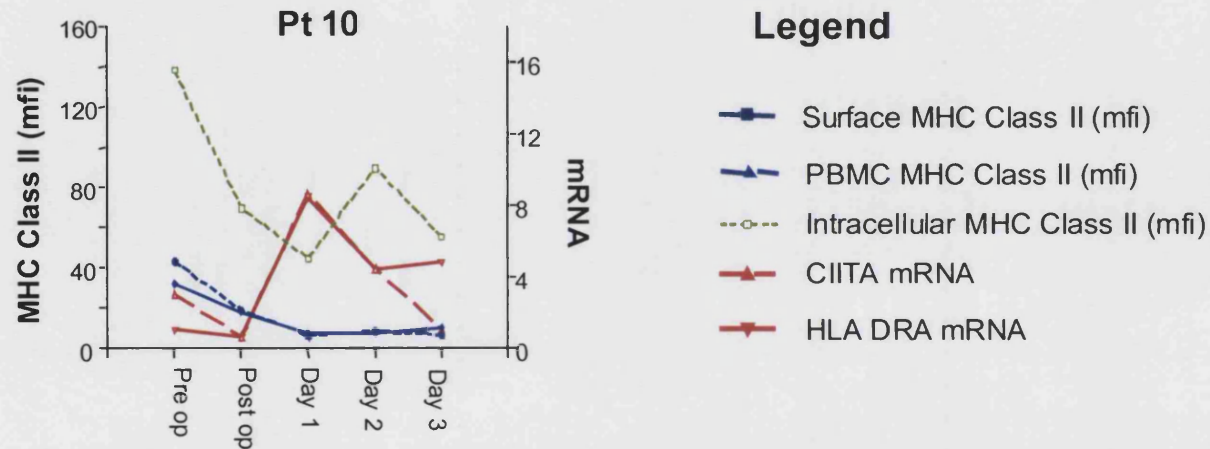


Figure 7.17 MHC Class II expression (mfi) and mRNA levels for 10 patients. Each patients MHC Class II surface expression on monocytes from whole blood and PBMC's is plotted along with intracellular MHC Class II (left axis). The quantitative values of messenger RNA for HLA-DRA and CIITA are plotted in red. In all but two patients (pt 1 & 8) there was an increase in mRNA levels following surgery. This increase in mRNA preceded an increase in the intracellular staining for MHC Class II. There was no Day 3 mRNA sample for Patient 5. For simplicity, axes are only labelled on patient 1-3 and 10. These axis apply to all graphs presented in this figure.

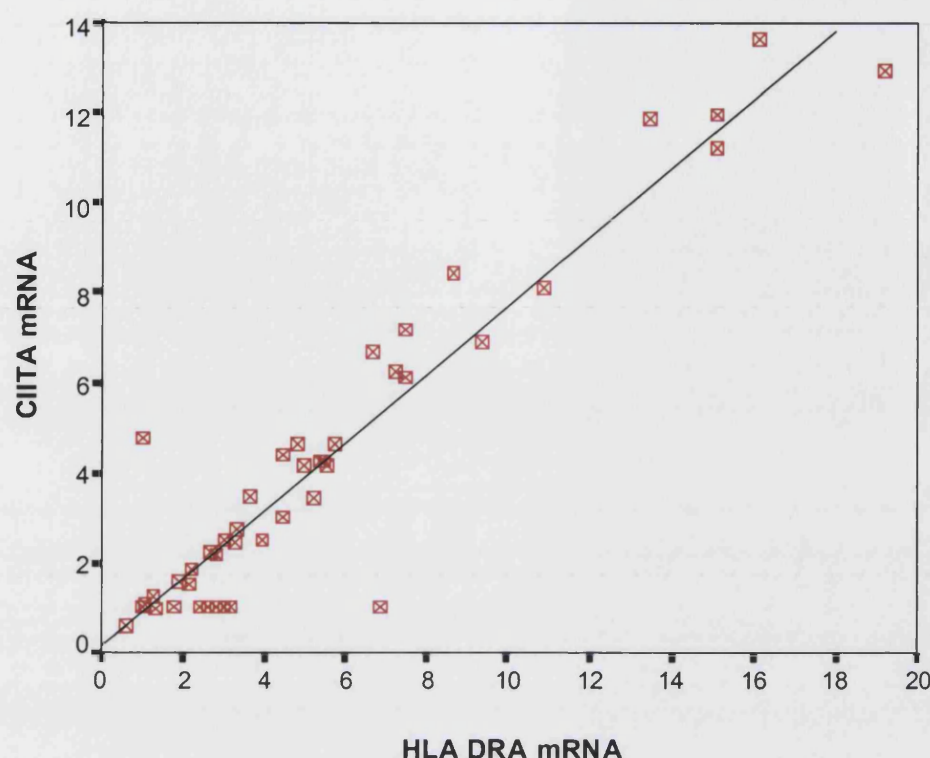


Figure 7.18 Levels of HLA-DRA mRNA pre and post-operatively tightly correlated with levels of CIITA mRNA. (Spearman correlation co-efficient $r=0.95$, $p<0.0001$).

7.3.2.5 MHC Class II expression, mRNA levels and circulating cytokines

Systemic cytokine levels were measured in this study group because of the intimate relationship reported between $\text{TNF-}\alpha$, IL-10 and MHC Class II expression (Fig 7.19). The overall cytokine profile did not differ from the results presented in Chapter 4 where a larger cohort of cardiac patients was examined. All 10 patients had a significant rise from pre-operative levels of IL-6 and IL-8 at the end of surgery ($p<0.001$ Mann-Whitney). Information gained from Chapter 4 would suggest that peak levels of both cytokines may have been missed as no samples were collected between the end of cardiopulmonary bypass and 0900 the next morning. Systemic levels of IL-6 and IL-8 remained significantly elevated on Day 3 ($p<0.01$). Levels of IL-6 and IL-8 peaked between 70 and 325 pg/ml and 36 and 225pg/ml respectively which were lower than peak levels recorded by ELISA in the Chapter 4 cohort.

In both this study and that presented in Chapter 4, peak levels of IL-10 were seen at the end of surgery following modified ultrafiltration. All 10 patients studied here had a significant rise in IL-10 from pre-operative levels during theatre ($p < 0.001$). IL-10 levels at the end of surgery ranged from 20 to 335 pg/ml, with a median of 112.8 pg/ml. This was higher than the median IL-10 level seen in Chapter 4 at the same time point (end of modified ultrafiltration: median 67 pg/ml).

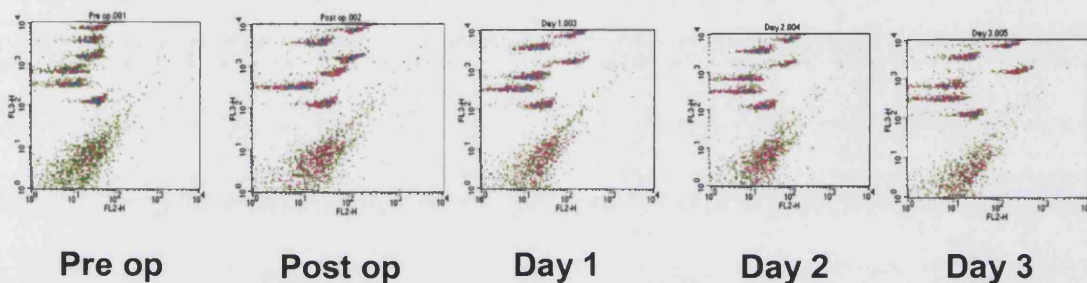


Figure 7.19 Appearance of plasma cytokine data for one patient. At the end of theatre, IL-8, -6 and -10 are all increased from pre-operative levels. IL-8 and -6 remain elevated on the 3rd post-operative day. FL2-H mfi is read against a standard curve to calculate cytokine concentration.

The peak levels of IL-10 at the end of surgery did not correlate with duration of bypass, aortic cross-clamp, or circulatory arrest time, however they did negatively correlated with the lowest intra-operative temperature (Fig 7.20). The height of the peak of circulating IL-10 did not correlate with the degree of reduction in surface MHC Class II expression seen in the post-operative period, nor did it appear to have a direct relationship to levels of HLA-DRA or CIITA mRNA levels.

TNF- α levels were largely undetectable in this patient population using this method, so no assessment of the role of TNF- α as an inducer of transcription of MHC Class II related genes could be made.

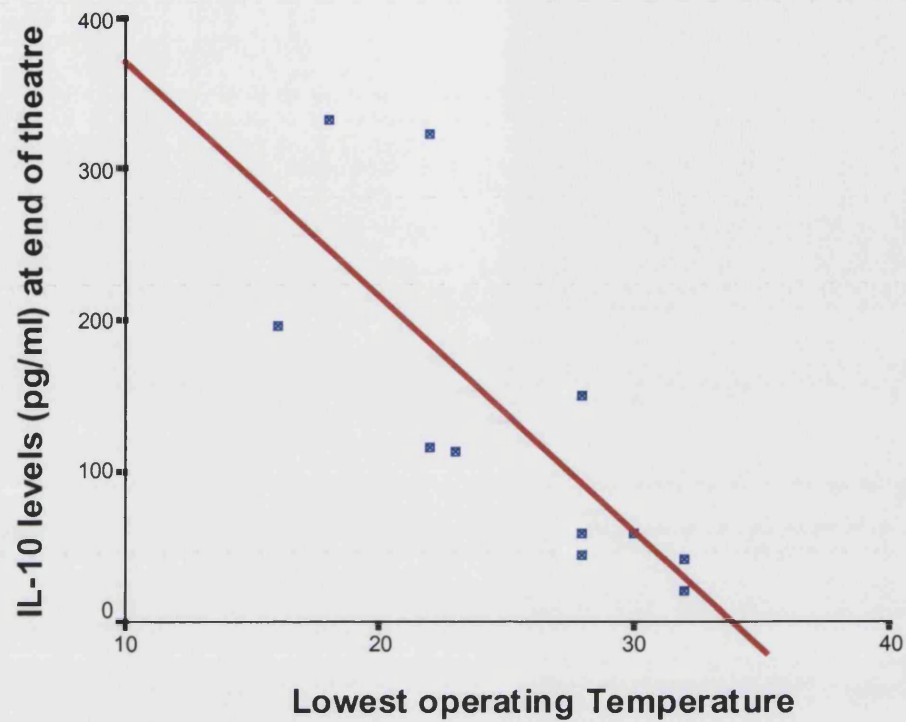


Figure 7.20 Levels of IL-10 at the end of surgery negatively correlated with the operating temperature chosen for the surgical repair. ($r = -0.78$, $p = < 0.005$). There was no correlation between IL-10 levels at the end of theatre and CPB time, circulatory arrest time, or length of stay.

7.4 DISCUSSION

A reduction in surface MHC Class II expression on monocytes from children following cardiac surgery was shown in Chapter 3 to be a significant independent risk factor for the development of post-operative complications. There are now several studies confirming that an inflammatory insult results in a reduction of surface MHC Class II on monocytes, and that the degree and persistence of reduction is a predictive parameter for the incidence of sepsis and/or clinical outcome (Cheadle, Hershman and others, 1991; Giannoudis, Smith and others, 1999; Hershman, Cheadle and others, 1990; Klava, Windsor and others, 1997; Tschakowsky, Hedwig-Geissing and others, 2002; van den Berk, Oldenburger and others, 1997; Wakefield, Carey and others, 1993). The molecular mechanisms underlying this fall in surface expression of MHC Class II is at present poorly understood. *In vitro* studies suggest that IL-10 and TGF- β play a role by blocking exocytosis and recycling of intracellular molecules (Astiz, Saha and others, 1996; Docke, Randow and others, 1997; Randow, Syrbe and others, 1995). There has been a single clinical study in septic adults that suggested reduction in surface MHC Class II expression was a post-translational effect and that this was partially mediated by circulating levels of IL-10 (Astiz, Saha and others, 1996). The findings of the work presented above raise an alternative hypothesis.

Results from the 10 patients in this study suggest that monocyte HLA-DR and CIITA mRNA production is normally at a low level, and that this is sufficient to maintain high levels of surface MHC Class II expression and excessive amounts of intracellular HLA-DR. Following the inflammatory insult of cardiac surgery and CPB, surface expression of MHC Class II (both % and mfi) fell. Associated with this was a reduction in intracellular MHC Class II median fluorescent staining in 9 out of 10 patients, and an increase in mRNA levels for HLA-DR and CITTA. The relative increase in gene transcription was not clearly related to the degree of fall in surface or intracellular expression of MHC Class II (Fig 7.17), nor was it related to measurable systemic levels of TNF- α or IL-10.

These finding would argue against the hypothesis that the fall in surface MHC Class II expression following CPB is due to re-endocytosis and cellular sequestration of the

greatest risk of post-operative complications following cardiac surgery. This was particularly true for sepsis.

8.2.2 Children have reduced capacity to respond to a second inflammatory insult in the immediate period following cardiac surgery

It has also become apparent from this work, that all children enter a state of *immune paresis* in the immediate post-operative period as demonstrated by reduced whole blood cytokine response to LPS stimulation. This state persists for at least 48 hours. This means that during the early post-operative period, children may not be able to mount an appropriate inflammatory response to a further insult and particularly to infection. During the early post-operative period patients are at particular risk of a number of such insults which include infection from an indwelling catheter, translocation of bacteria from the GI tract, poor tissue perfusion, haemorrhage and barotrauma from excessive ventilation. These may also be sufficient to propagate an excess anti-inflammatory state further increasing the risk of nosocomial sepsis, multi-organ failure and death. This 'multi-hit' model where CPB primes the patient but a secondary insult in the post-operative period is required to drive the loss of homeostasis would fit nicely with the clinical picture.

As monocytes are the major circulating cell producing cytokines, it has been postulated here, but not proven, that the LPS hypo-responsiveness is due to monocyte *deactivation*. Whilst the degree of LPS hypo-responsiveness could not be directly related to the degree of reduction in MHC Class II expression, these two events were related in time, and may have a similar underlying aetiology. *In vitro* studies (Brandtzaeg, Osnes and others, 1996; Dehoux, Hernot and others, 2000; Grundmann, Rensing and others, 2000; Randow, Syrbe and others, 1995) would suggest that IL-10 is a potential candidate. In support of this, the studies undertaken here show that circulating levels of IL-10 significantly increased following CPB. Another candidate is TGF beta (Randow, Syrbe and others, 1995).

Interleukin-10 has been shown to reduce MHC Class II surface expression on the monocytes of septic patients at a post-transcriptional level, by impeding recycling of surface MHC Class II and exocytosis of newly formed MHC Class II, with no change to

the mRNA levels (Fumeaux and Pugin, 2002). The results presented in Chapter 7 questions whether this is the mechanism behind the reduction in MHC Class II in cardiac surgical patients. The fall in surface expression of MHC Class II on monocytes was paralleled by a fall in intracellular MHC Class II (mfi) staining. This was confirmed with confocal microscopy. Twenty-four to forty-eight hours following the reduction in surface and intracellular MHC Class II staining, an increase in transcription of genes required for production of MHC Class II was observed. These observations suggest an alternate hypothesis for the reduction in MHC Class II seen following CPB. Secretion or cleaving of surface bound MHC Class II molecules maybe occurring during surgery. Depletion of intracellular stores occurs, presumably in order to replenish surface expression, but appears to be ineffective during the first 72 hours following surgery. At this stage increased transcription occurs but only leads to greater MHC class II expression in patients who are recovering.

8.2.3 Genetic predisposition plays a role in determining outcome

Whilst the inflammatory insult delivered can be stratified and has the potential for modification, the host response to this insult is at present an unknown. Certainly younger age, and co-morbid conditions are associated with a worse outcome (Brown, Ridout and others, 2003), however genetic factors which modify the host's inflammatory response are also likely to play an important role in determining outcome. From the work undertaken in this thesis it appears that the presence of polymorphisms in the IL-6 and TNF- α promoters have a significant association with duration of ventilation and/or length of stay. Whether this is through a direct effect on cytokine levels produced in response to an insult, or the result of a more complicated process remains to be determined. It is interesting that these results differ from those found in some adult studies and further work will be required to clarify the reasons why.

8.3 LIMITATIONS OF THIS WORK

8.3.1 Confounding variables

There are many limitations of the work presented here. Some of these are inevitable in clinical studies, and particularly those performed in children. While studies of the type

undertaken here introduce many potential confounders, interestingly, environmental factors are less likely to confound paediatric studies. This should enable potential genetic contributions to be accurately assessed. That said, the heterogeneity of the congenital cardiac defect population is still restrictive and only large multicentre studies will eliminate this as a confounder.

8.3.2 Considering monocytes in isolation

Much of the data presented in this thesis emanates from studies performed in whole blood. An assumption has been made that the changes observed are reflecting changes in monocytes. However monocytes are only one group of cells operating in an extremely complex inflammatory environment. As such there is an inherent risk of over interpretation of the data.

Circulating monocytes and plasma levels of cytokines were examined in detail during this project. Whilst peripheral blood is relatively easy to obtain, it is distal to the site of primary insult. Results presented here may not reflect local tissue levels of inflammatory mediators or local cytokine balance. The cardiac myocyte may be exposed to far greater cytokine peaks than are evident from analysis of plasma. However the likelihood of designing a study acceptable to an ethics committee that examines cardiac tissue or coronary sinus blood is remote in children.

Monocytes have a short life span in the circulation ($t_{1/2} = 17$ hours). Therefore one possible explanation for the findings is that changes in MHC Class II represent changes solely in the circulating population of monocytes. There are features of this work which argue against this being so. For example if the inflammatory stimulus of surgery resulted in all monocytes with high MHC Class II surface expression leaving the circulation, one would expect a significant fall in the number of circulating monocytes. This did not occur (Fig 5.3). In addition the findings of increased transcription of MHC Class II related genes in monocytes with reduced surface and intracellular expression of MHC Class II 48-72 hours post-operatively, supports the view that the changes in MHC class II was common to more than just the circulating population of monocytes.

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shedding or shearing of MHC Class II from the surface of the monocyte contrasts with findings in septic patients. The pilot work presented here could be supplemented at several levels. Absolute quantitation of the transcriptional products from the MHC related genes could be performed using RT-PCR. Changes in intracellular staining on flow cytometry and confocal microscopy could be further supported by quantitating the intracellular protein content of the monocyte with a technique such as western blotting. Plasma from patients pre and post-operatively could be examined using flow cytometry and ELISA for unbound MHC Class II. Isolated bypass circuits primed with whole blood could be used to examine changes in surface MHC Class II expression due to mechanical stress.

These experiments will not however identify the mediators responsible for changes in MHC Class II expression. To investigate this further, design may necessitate the use of validated animal models of bypass, and monocytic cell lines so that local levels of mediators can be determined and blocking antibodies trialled.

8.6.2 Validating the findings of a genetic predisposition to outcome

Additional work has already commenced to validate the genetic findings presented here. All patients admitted to CICU following surgery involving CPB are being genotyped for the cytokine polymorphisms investigated above, as well as MHC genotyping. In addition plasma has been collected daily for the first 3 days to assess plasma cytokine levels. Collaboration with an American paediatric cardiac centre will enable validation of results on another patient cohort. Larger study numbers will enable the genetic contribution to outcome to be further assessed by construction of logistic regression models such as those described in Chapter 3.

There are no published studies looking for genetic factors which may influence the degree of reduction in MHC Class II expression that occurs following an inflammatory insult. In Chapter 6 changes in surface MHC Class II were not examined. Regulation of MHC Class II expression is in part controlled by local cytokine levels. The presence of a promoter polymorphism that is associated with high levels of IL-10 could be hypothesised to result in an exaggerated fall in MHC Class II expression in the post-operative period, with the reverse being true for the TNF- α promoter polymorphism.

The response of monocyte surface expression may also be influenced by the MHC haplotype, especially as it is in linkage disequilibrium with other inflammatory genes.

8.6.3 Targeted immunomodulation

The strong predictive value of post-operative MHC Class II surface expression in the immediate post-operative period and the subsequent development of sepsis/SIRS raise the possibility of therapeutic immunomodulation. At present there is no immune monitoring or treatment strategy for patients in cardiac intensive care. Monitoring MHC Class II surface expression and whole blood responsiveness to LPS stimulation over the first 72 post-operative hours enables identification of the subgroup of patients most likely to develop post-operative complications including sepsis/SIRS. Combining this laboratory result with a clinical assessment that the patient is unlikely to leave the unit in the following 24 hours, would result in selecting a target population for immunomodulation. The most promising candidate for immune modulation would be GM-CSF. GM-CSF has been shown to stimulate haematopoiesis resulting in an increased granulocyte count, increase MHC Class II expression and restore the antigen presenting potential on monocytes (Flohe, Borgermann and others, 1999; Mellstedt, Fagerberg and others, 1999; Perry, Mostafa and others, 2003). Evidence for the clinical use of GM-CSF suggests that it is well tolerated even in neonates (Bilgin, Yaramis and others, 2001; Presneill, Harris and others, 2002). The hypothesis would be that by selecting the group of patients with both *immune paresis* and a requirement for ongoing intensive care support, treatment with GM-CSF would result in an increase in both circulating monocyte count and MHC Class II expression, a reduction in the septic complications, and a reduction in mortality.

8.6.3 Mathematical modelling

The nature of the immune system is extremely complex with many interactions between systems. Clinical studies are 'muddled' by confounders and the inability to control for the other immunological mediators. *In vitro* work equally has limitations. Whilst cell work enables the researcher to isolate the cell of interest, that in itself may cause misleading results. One possibility to maximise scientific gains from both fields of research would be to embark on mathematically modelling the inflammatory response

to bypass. This is complex but may provide insights into pathogenesis not possible through other means.

8.7 CURRENT CONCEPTUAL MODEL

From the results obtained from this thesis combined with those reported in the existing literature it is possible to map out potential inflammatory sequences which could explain the observed clinical course in children undergoing cardiac surgery. This is described below and in figure 8.1.

Cardiac surgery and CPB elicits a profound systemic inflammatory response in children. This may to some extent be genetically determined. After surgery, the inflammatory system attempts to restore homeostasis, and in doing so passes from an excessive pro-inflammatory response in the first few hours to an excessive anti-inflammatory response over the first few days. This can be monitored by the reduction in monocyte MHC Class II expression. Any second insult that occurs may propagate the see-sawing of the immune system and prevent the return of homeostasis. Under such circumstances a series of minor sequential insults may result in the persistent immune imbalance, overt sepsis, development of multi-organ dysfunction and late death.

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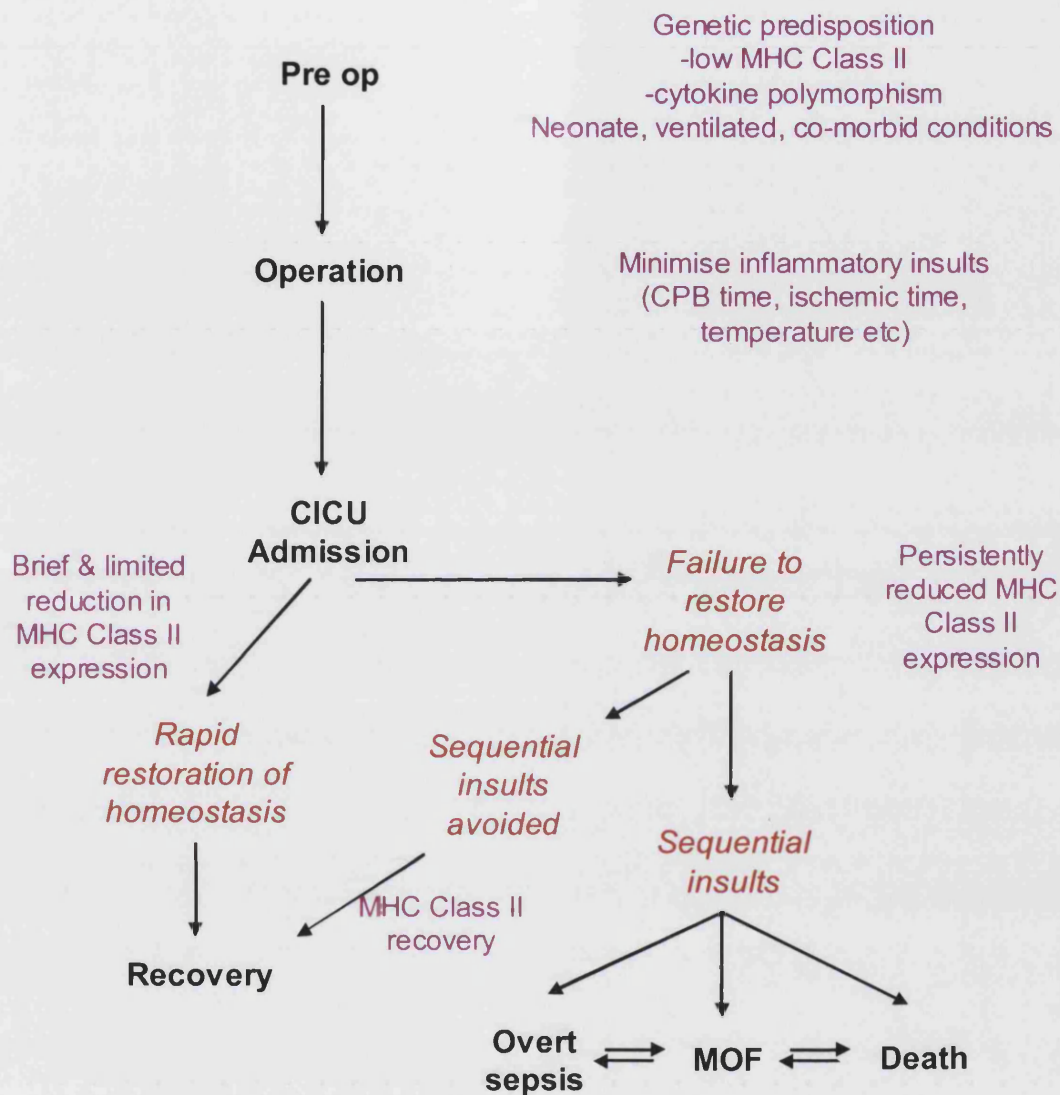


Figure 8.1 Outline of possible pathways a child may take following cardiac surgery. Pre-operatively the child may be at particular risk of post-operative complications because of age, requirement for assisted ventilation prior to surgery, or have associated co-morbid conditions. In addition, the child's inflammatory response to surgery may be influenced genetically. There are features of the operation, such as prolonged bypass and ischemic time which suggest risk of a complicated post-operative period. When the patient is admitted into CICU, the inflammatory system will be attempting to regain homeostasis. Initially all children will have a fall in their MHC Class II surface expression on monocytes. If homeostasis is achieved, the MHC Class II expression will be normalising in the first few post surgical days. If homeostasis is not achieved, the MHC Class II expression may remain reduced, and the child may be at particular risk of secondary infection and organ dysfunction. If further inflammatory insults can be avoided, homeostasis may return and the patient may recover. If sequential inflammatory insults can not be avoided then immune imbalance may persist, and the patient is at high risk of significant post-operative morbidity and/or mortality.

APPENDIX I

Phenotypic data collected on each patient

Demographics	Operative details	CICU Course
Date of birth	Date and time of operation	Dat and time of admission
Gestation	Weight	PIM score
Race	Operation performed	Risk of Mortality score
Gender	Operator	Duration of intubation
Cardiac diagnosis	Anaesthetic agents used	Total time in CICU
Age at diagnosis	Lowest temperature	Outcome at 28 days
Previous interventions	CPB time	Inotropes
Co-morbid conditions	X-clamp time	Rhythm disturbances
Recent infection	Circ arrest time	Chest open
Invasive proceedure in last 2 weeks	Dexamethasone	Bleeding
Saturations pre-op	Cardioplegia solution	Reexploration
Shunt pre op	Pre op Hct	Sepsis – organism / site
	Post op Hct	SIRS
		Line sepsis
		DIC
		Renal failure
		Liver failure
		Other complications

APPENDIX II

Paediatric Index of Mortality (PIM Score)

Variables	Values (1 if Yes, 0 otherwise)	Beta
Elective admission	<input type="text" value="0"/>	0
Underlying condition	<input type="text" value="0"/>	0
Response of pupils to bright light (> 3 mm and both fixed)	<input type="text" value="0"/>	0
Mechanical ventilation (at any time during first hour in ICU)	<input type="text" value="0"/>	0
Systolic Blood Pressure (mmHg)	<input type="text" value="120"/>	0.021
Base excess (mmHg) (arterial or capillary blood)	<input type="text" value="0"/>	0.071
FiO2 (%) / PaO2 (mmHg)	<input type="text" value="0"/>	0.415

Predicted Death Rate :
([help](#))

Logit = (-4.873) + (values * Beta) + (0.021 * (absolute(SBP-120))) + (0.071 * (absolute base excess)) + (0.415 * (FiO2/PaO2))

Predicted death rate = $e^{\text{Logit}} / (1 + e^{\text{Logit}})$

APPENDIX III

American College of Chest Physicians / Society of Critical Care Medicine Consensus Conference 1992

Abbreviated definitions of Sepsis and the systemic inflammatory response syndrome (SIRS)	
<i>SIRS</i> The systemic inflammatory response to a variety of severe clinical insults. Includes 2 or more of the following:	
Temperature	>38 C (100.4 F) or <36 C (96.8 F)
Heart rate	>90 beats/min
Respiratory rate	>20 breaths/min or Paco_2 <32 torr (<4 kPa)
White blood cell count	>12,000 cells/mm ³ , <4000 cells/mm ³ or > 10% band cells
<i>Sepsis</i> The systemic response to infection . The response is manifest by the same criteria as SIRS	
<i>Severe sepsis</i> Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include but are not limited to lactic acidosis, oliguria, or an acute alteration in mental status.	
<i>Septic shock</i> Sepsis associated with hypotension, despite adequate fluid resuscitation, along with the perfusion abnormalities listed for severe sepsis. Patients who are on inotropic or vasopressor agents need not be hypotensive at the time perfusion abnormalities are measured.	

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